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The cleavage of pyrophosphate by human serum apo-transferrin

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[**ABSTRACT**] **Objective**: To determine the rate constants for the cleavage of pyrophosphate to phosphate by human serum apo-transferrin. **Methods**: The cleavage was followed by ^{31}P NMR spectroscopy. The data for concentration changes of pyrophosphate (as determined from its NMR peak intensity) with time, in the absence and the presence of $MgCl_2$, at different pH values, were fitted to give second order rate constants. **Results**: The rate constants at 312 K were 8.83×10^{-4} L·mmol^{-1}·h^{-1} at pH 6.85, 9.59×10^{-4} L·mmol^{-1}·h^{-1} at pH 7.4, and 1.38×10^{-3} L·mmol^{-1}·h^{-1} at pH 8.15, for reactions of apo-transferrin (0.5-1.0 mmol/L) with 5 molar equivalence of pyrophosphate. The rate constant increased to 1.21×10^{-3} L·mmol^{-1}·h^{-1} at pH 7.4, 312 K when 2 mmol/L $MgCl_2$ was added. **Conclusion**: The cleavage of pyrophosphate to phosphate by human serum apo-transferrin is very slow and follows the second-order kinetics. Mg^{2+} can slightly enhance the rate of the cleavage.

[**KEY WORDS**] pyrophosphate; human serum apo-transferrin; cleavage; rate constants

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Phosphorylation is generally considered the most frequent and universal post-translational modification of proteins involved in the regulation of various biological processes. Pyrophosphate can serve as a source of phosphoryl groups for the phosphorylation of proteins in a variety of living organisms[1-3]. In the presence of Mg^{2+} the protein-bound pyrophosphate is rapidly exchanged with phosphate in the medium[4]. The cleavage of pyrophosphate linkages is important in the enzymic process of pyrophosphatase, in the biosynthesis of nucleotides, and in the energy support of biological reactions.

Pyrophosphate is one of the commonly used chelators for the removal of Fe^{3+} from transferrins. Human serum transferrin (hTF), a member of the transferrin superfamily which contains lactoferrin, ovotransferrin, and bacterial ferric-ion binding protein, is the serum iron transport protein. Its primary functions are the uptake and binding of Fe^{3+} in specific binding sites, and the release of Fe^{3+} to cells *via* receptor-mediated endocytosis at low pH[5]. hTF is then recycled as apo-hTF back to the surface of the cell (100-200 recycles of reversible Fe^{3+} uptake and delivery during its lifetime in circulation[6]). X-ray crystallographic studies on full-length or recombinant N-lobe/C-lobe human transferrins[7-9] and other transferrins[5] have revealed that hTF is a single-chain glycoprotein (M_W *ca.* 80 000) folded into 2 globular lobes (N- and C-lobe), each of which contains a distorted octahedral Fe^{3+} binding site consisting of 2 Tyr, one His and one Asp, and a bidentate carbonate (the synergistic anion) as ligands.

The protein can bind and transport a range of other metal ions as well as chemotherapeutic drugs[5,10-13]. Only *ca.* 30% of the specific Fe^{3+} binding sites are saturated with Fe^{3+} in the serum and there are high levels of transferrin receptors on the surface of tumour cells[14].

The binding kinetics and binding affinity of a variety of monovalent and divalent inorganic anions, including pyrophosphate, phosphate, and bicarbonate, have been investigated for elucidation of the mechanism of metal binding and release[15-17]. The binding constants for pyrophosphate to apo-hTF have been determined to be logK_1 6.23 and logK_2 4.67 by UV-visible spectroscopy, which are the

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biggest among those inorganic anions tested. For phosphate the equivalent figures are logK_1 4.65 and logK_2 4.01, which are bigger than those for bicarbonate (logK_1 3.00 and logK_2 2.30[15]), although (bi) carbonate is the only inorganic anion which functions as a synergistic anion in forming an Fe^{3+}-anion-transferrin ternary complex[18].

Although serum transferrin is thought to function as an iron transport protein, we are interested in the possibility that it may also have other functions. Here we have shown that it has phosphatase activity.

1 MATERIALS AND METHODS

1.1 Materials Sodium pyrophosphate decahydrate was purchased from Strem Chemicals. Fe atomic absorption standard solution (1 000 μg/ml Fe in 3% HNO_3), 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (Hepes), human serum apo-transferrin (apo-hTF, catalog No. T1147) and holo-hTF (catalog No. T4778) were from Sigma-Aldrich.

Low molecular mass impurities in apo-hTF were removed by ultrafiltration for 3 times with 10 mmol/L Hepes buffer pH 7.4, using a concentrator (cutoff 30 000, VIVASCIENCE) to give a final stock solution of *ca.* 1 mmol/L concentration. The concentration of apo-hTF was determined by UV-visible spectroscopy according to the extension coefficient $\varepsilon = 93\ 000\ L \cdot mol^{-1} \cdot cm^{-1}$ at 280 nm[19].

1.2 NMR spectroscopy ^{31}P NMR spectra were acquired in 5 mm NMR tubes on a Bruker DMX 500 NMR spectrometer at 202 MHz with a TBI probe-head or on a DPX 360 NMR spectrometer at 145.8 MHz with a QNP probe-head, and equipped with z-field gradients, using inverse-gated ^1H decoupling. Typically 1D ^{31}P-{^1H} NMR spectra were acquired with 512 transients into 16 384 data points over a spectral width of 5.8 kHz (40) using a relaxation delay of 0.6 s, at 312 K with 1 h, 2 h or 5 h intervals. NMR data processing was carried out on a Silicon Graphics computer using XWIN NMR software (Version 3.1, Bruker).

All samples for ^{31}P NMR spectroscopy were in 10 mmol/L Hepes buffer containing 10% D_2O. Solutions of *ca.* 0.6 ml 0.5-1.0 mmol/L apo-hTF, into which an appropriate volume of 200 mmol/L pyrophosphate (reaction molar ratio of pyrophosphate: apo-hTF = 5 : 1) was added. The pH was finally adjusted to 6.85, 7.40, or 8.15 with 0.9 mol/L $HClO_4$ or KOH. The mixture of 5 mmol/L pyrophosphate in Hepes buffer with or without 1/60 mol equivent Fe^{3+} (by adding microlitre aliquots of Fe atomic standard solution, and finally adjusting pH to 7.4) were used as reference standards.

1.3 Kinetics measurements The second-order rate constants (k) for the cleavage of pyrophosphate by apo-hTF at pH 6.85, 7.40, and 8.15, 312 K were obtained by fitting the curves describing the concentrations of pyrophosphate (PPi) at different times according to equation (1):

$$\frac{1}{c_{PPi}} = -kt + \frac{1}{c_0} \qquad (1)$$

where c_0 is the initial concentration of pyrophosphate (at time 0) and c_{PPi} the concentration at time t. c_{PPi} was calculated using the following equations:

$$\frac{c_{Pi}}{c_{PPi}} = f_r \qquad (2)$$

$$\frac{1}{2}c_{Pi} + c_{PPi} = c_0 \qquad (3)$$

where c_{Pi} is the concentration of phosphate formed during the cleavage process and f_r is the ratio of the (arbitrary) volume integral of the peaks for pyrophosphate *vs* phosphate on the ^{31}P NMR spectra.

1.4 pH measurements The pH values of samples were measured at 298 K using a Corning 240 pH meter equipped with an Aldrich micro combination electrode calibrated with Aldrich buffer solutions at pH 4, 7, and 10. The pH values of NMR samples in 10 mmol/L Hepes buffer/D_2O 9 : 1 were measured directly in the NMR tube and no correction has been applied for the effect of deuterium on the glass electrode.

2 RESULTS

2.1 Cleavage of pyrophosphate by human serum apo-transferrin The cleavage of pyrophosphate by human serum apo-hTF was followed by ^{31}P NMR spectroscopy. On the ^{31}P NMR spectra of apo-hTF with pyrophosphate in 10 mmol/L Hepes buffer pH 7.4 at 312 K, the intensity of the peak for pyro-

phosphate (−6.59) decreased and shifted to a slightly higher frequency with time and a new peak appeared at 2.74, corresponding to phosphate, with increasing intensity (Fig 1). The ratios of the (arbitrary) peak integrals for pyrophosphate *vs.* phosphate were used to calculate the concentrations of pyrophosphate, and the latter in turn were best fitted with equation (1), to yield the second-order rate constant $k=(9.59\pm0.10)\times10^{-4}$ L · mmol^{-1} · h^{-1} ($r=0.999$) (Fig 2, Tab 1). The rate constant increased to 1.38×10^{-3} L · mmol^{-1} · h^{-1} when the pH was raised to 8.15, but decreased to 8.83×10^{-4} L · mmol^{-1} · h^{-1} when the pH dropped to 6.85 (Tab 1).

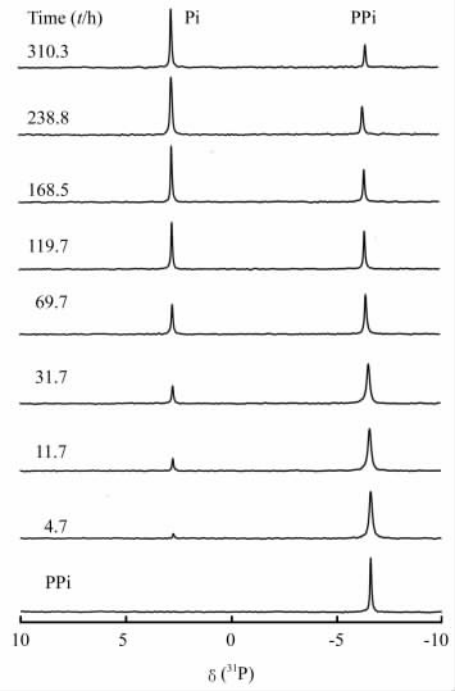

Fig 1 ^{31}P NMR spectra acquired at 202 MHz for the reaction of pyrophosphate with apo-hTF at different times

The reaction was carried out in 10 mmol/L Hepes/D_2O (9 : 1) at pH 7.4, 312 K at a mol ratio of pyrophosphate : apo-hTF (0.93 mmol/L) of 5 : 1. The spectra were recorded at 0.4, 7.7, 11.7, 31.7, 69.7, 119.7, 168.5, 238.8, and 310.3 h. PPi and Pi represent pyrophosphate and phosphate, respectively

2.2 Catalysis on cleavage of pyrophosphate by $MgCl_2$ Catalysis with Mg^{2+} in the cleavage process of pyrophosphate by apo-hTF was tested when $MgCl_2$ (final concentration 2 mmol/L) was present. The rate constant became 1.21×10^{-3} L · mmol^{-1} · h^{-1} at 312 K, which was larger when compared with the reaction without $MgCl_2$.

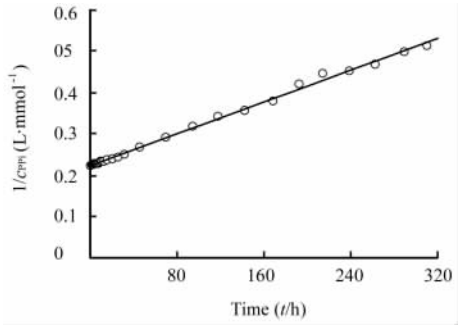

Fig 2 Decrease in concentration of pyrophosphate with time for reaction of pyrophosphate with apo-hTF

○: $1/c_{PPi}$; —: Second-order fit curve. Reaction conditions are the same as for Fig 1

Tab 1 Second-order rate constants for cleavage of pyrophosphate by transferrin

Reaction mixture	Conditions	k (v/L · mmol^{-1} · h^{-1})	Correlation coefficient
apo-hTF+PPi	pH 6.85	$(8.83\pm0.56)\times10^{-4}$	0.950
apo-hTF+PPi	pH 7.40	$(9.59\pm0.10)\times10^{-4}$	0.999
apo-hTF+PPi	pH 8.15	$(1.38\pm0.58)\times10^{-3}$	0.965
apo-hTF+PPi	pH 7.40, 2 mmol/L $MgCl_2$	$(1.21\pm0.79)\times10^{-3}$	0.960

All reactions were carried out in 10 mmol/L Hepes/D_2O (9 : 1), 312 K. The reaction ratios of pyrophosphate (PPi) : apo-hTF (0.5-1.0 mmol/L) are 5 : 1

3 DISCUSSION

The cleavage of pyrophosphate is important in many biological reactions, including the enzymic process of pyrophosphatase, the biosynthesis of nucleotides, and the energy support for biological systems. Pyrophosphate is also a source of phosphoryl groups for protein phosphorylation in a variety of living organisms[1-3]. The binding of pyrophosphate to apo-hTF is reported stronger than that of (bi) carbonate, the synergistic anion for the binding of Fe^{3+} to transferrins, by UV-visible spectroscopy[16].

The reaction of pyrophosphate with human serum apo-hTF resulting in a release of phosphate was observed by ^{31}P NMR spectroscopy (Fig 1). Observed from the ^{31}P NMR spectra, the intensity of the peak for pyrophosphate decreased and shifted to a slightly higher frequency with time. This indicates that pyrophosphate is involved in a fast exchange process between its free and bound form in

which the equilibrium is slowly shifting. Best fitting of the concentration of pyrophosphate with time gave rise to a second-order rate constant of 9.59×10^{-4} L · mmol^{-1} · h^{-1} at pH 7.4，312 K (Fig 2，Tab 1). The hydrolysis of pyrophosphate itself in the absence of the protein is very slow and no obvious release of phosphate was observed within 17 d under the same reaction conditions (data not shown). The cleavage is dependent on pH with an increasing rate constant at high pH，suggesting that the deprotonation of tyrosine in the protein could be important to the process. In the UV-visible spectroscopic difference spectra，the reaction of pyrophosphate with apo-transferrin resulted in negative peaks at 245 nm and around 300 nm (data not shown)，indicating that the phenolate groups of tyrosines，the specific Fe^{3+} binding sites，are also the binding sites for pyrophosphate[16]. However，on the basis of our ^{31}P NMR measurements it was found that the free amino acid tyrosine itself cannot cleave pyrophosphate to release phosphate under the experimental conditions used (data not shown).

When $MgCl_2$ was present the rate constant increased by *ca*. 1.3 fold compared with that when $MgCl_2$ was abscent. This result reveals that cleavage of pyrophosphate to phosphate by apo-transferrin can be catalysed by Mg^{2+} although the catalysis is weak.

[REFERENCES]

[1] Duclos B, Vaganay E, Dadssi M, et al. Pyrophosphate is a source of phosphoryl groups for *Escherichia coli* protein phosphorylation[J]. FEMS Microbiol Lett，1996，145：49-54.

[2] da-Silva LP，Lindahl M，Ludin M，et al. Protein phosphorylation by inorganic pyrophosphate in yeast mitochondria[J]. Biochem Biophys Res Commun，1991，178：1359-1364.

[3] Pramanik AM，Bingsmark S，Lindahl M，et al. Inorganic-pyrophosphate-dependent phosphorylation of spinach thylakoid proteins[J]. Eur J Biochem，1991，198：183-186.

[4] Bakuleva NP，Nazarova TI，Baykov AA，et al. The phosphorylation of yeast inorganic pyrophosphatase and formation of stoichiometric amounts of enzyme-bound pyrophosphate[J]. FEBS Lett，1981，124：245-247.

[5] Sun H, Li H，Sadler PJ. Transferrin as a metal ion target[J]. Chem Rev，1999，99：2817-2842.

[6] Katz JH. Iron and protein kinetics studied by means of doubly labeled human crystalline transferrin[J]. J Clin Invest，1961，40：2143-2152.

[7] Jeffrey PD, Bewley MC, MacGillivray RT, et al. Ligand-induced conformational change in transferrins：crystal structure of the open form of the N-terminal half-molecule of human transferrin[J]. Biochemistry，1998，37：13978-13986.

[8] MacGillivray RT，Moore SA，Chen J，et al. Two high-resolution crystal structures of the recombinant N-lobe of human transferrin reveal a structural change implicated in iron release[J]. Biochemistry，1998，37：7919-7928.

[9] Zuccola HJ. The crystal structure of monoferric human serum transferrin[D]. Atlanta：Georgia Institute of Technology，1993.

[10] Zhong W，Parkinson JA，Guo M，et al. Unusual features for zirconium(Ⅳ) binding to human serum transferrin[J]. J Biol Inorg Chem，2002，7：589-599.

[11] Guo M，Sun H，McArdle HJ，et al. Ti^{Ⅳ} uptake and release by human serum transferrin and recognition of Ti^{Ⅳ}-transferrin by cancer cells：understanding the mechanism of action of the anticancer drug titanocene dichloride[J]. Biochemistry，2000，39：10023-10033.

[12] Baker EN. Structure and reactivity of transferrin[J]. Adv Inorg Chem，1994，41：389-463.

[13] Li H，Sadler PJ，Sun H. Unexpectedly strong binding of a large metal ion (Bi^{3+}) to human serum transferrin[J]. J Biol Chem，1996，271：9483-9489.

[14] Panaccio M，Zalcberg JR，Thompson CH，et al. Heterogeneity of the human transferrin receptor and use of anti-transferrin receptor antibodies to detect tumors *in vivo*[J]. Immumol Cell Biol，1987，65：461-472.

[15] Harris WR，Cafferty AM，Trankler K，et al. Thermodynamic studies on anion binding to apotransferrin and to recombinant transferrin N-lobe half molecules[J]. Biochim Biochem Acta，1999，1430：269-280.

[16] Harris WR，Cafferty AM，Abdollahi S，et al. Binding of monovalent anions to human serum transferrin[J]. Biochim Biochem Acta，1998，1383：197-210.

[17] Harris WR，Nesset-Tollefson D. Binding of phosphonate chelating-agents and pyrophosphate to apotransferrin[J]. Biochemistry，1991，30：6930-6936.

[18] Foley AA，Bates GW. The influence of inorganic anions on the formation and stability of Fe^{3+}-transferrin-anion complexes [J]. Biochim Biochem Acta，1988，965：154-162.

[19] Harris DC. Different metal-binding properties of two sites of human transferrin[J]. Biochemistry，1977，16：560-564.

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人血清脱金属铁传递蛋白裂解焦磷酸键的研究

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[摘要] **目的**:测定人血清脱金属铁传递蛋白裂解焦磷酸键的反应速率常数。**方法**:应用^{31}P NMR 技术,在不同 pH 值及不同浓度 $MgCl_2$ 存在条件下,测定了脱金属铁传递蛋白与焦磷酸二钠反应的核磁共振图谱,根据焦磷酸盐的摩尔浓度(对应于其谱峰强度)随时间的变化情况,应用动力学公式对数据进行拟合。**结果**:当人血清脱金属铁传递蛋白(0.5~1.0 mmol/L)与焦磷酸盐的反应摩尔浓度比为 1∶5 时,在 312 K 条件下,反应速率常数分别为:8.83×10^{-4} L·mmol^{-1}·h^{-1}(pH 6.85)、9.59×10^{-4} L·mmol^{-1}·h^{-1}(pH 7.40)和 1.38×10^{-3} L·mmol^{-1}·h^{-1}(pH 8.15)。在 2 mmol/L $MgCl_2$ 存在时,pH 7.40、312 K 条件下,反应速率常数为 1.21×10^{-3}L·mmol^{-1}·h^{-1}。**结论**:人血清脱金属铁传递蛋白能缓慢地将焦磷酸根裂解为磷酸根,反应具有二级反应动力学性质,Mg^{2+} 对该裂解反应有弱催化作用。

[关键词] 焦磷酸盐;人血清脱金属铁传递蛋白;裂解;反应速率常数
[中图分类号] O 643 **[文献标识码]** A **[文章编号]** 0258-879X(2006)01-0071-05

Potent antitumoral efficacy of a novel replicative adenovirus CNHK300 targeting telomerase-positive cancer cells

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[**ABSTRACT**] PURPOSE: Telomerase reverse transcriptase (hTERT) is the key determinant of telomerase activity and plays a crucial role in cellular immortalization and oncogenesis. It will be a promising target for cancer gene therapy. We constructed a novel replicative adenovirus CNHK300 in which hTERT promoter with three extra E-boxes downstream of the promoter was introduced and used to regulate adenoviral E1a gene, and studied its properties of selective replication in cancer cells and antitumoral activity. METHODS: Luciferase assay was used to detect hTERT promoter activity. The selective replication of CNHK300 in cancer cells was investigated by E1a Western blot and green fluorescent protein (GFP) reporter gene assay. The antitumoral activity of CNHK300 and its toxicity were measured on animal models. RESULTS: Luciferase assay showed that introducing extra E-boxes downstream of hTERT promoter is beneficial to decreasing the promoter activity in normal cells without affecting its strong activity in cancer cells. Experiments *in vitro* and *in vivo* demonstrated that CNHK300 can selectively target to hTERT-positive cancer cells and replicate in them, resulting in oncolytic or antitumoral effect. CNHK300 is superior to ONYX-015 in terms of selective replication and oncolytic or antitumoral effect. The toxicity assay showed no signs of toxicity to liver cells even at the higher dosage of CNHK300 *in vivo*. CONCLUSION: The hTERT promoter-controlled, replication-competent adenovirus CNHK300 is a promising system for targeted cancer gene therapy.

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