CHEMISTRY LETTERS, pp. 21-24, 1980.

KINETICS OF THE SPECIFIC BINDING OF IRON(III)-NITRILOTRIACETATE TO HUMAN APO-TRANSFERRIN AND OF THE LIGAND EXCHANGE OF THE RESULTING COMPLEX USING THE STOPPED-FLOW TECHNIQUE

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The biphasicity was observed in the initial step of the reaction between Fe(III)-NTA and transferrin and it could be assigned not to the succesive mechanism involving two elementary steps of the reaction but to the difference in reactivity between two specific binding sites of transferrin.

The transferrins are a family of homologous iron(III)-binding protein with molecular weight of 70,000-80,000 present in blood serum, egg white and milky whey. They have two specific binding sites to which some transition metals can bind with different affinities. The relative affinities of two binding sites for iron(III) ion have been a nature of some controversy. An important aspect of the function of transferrin is the specific uptake and release of iron(III) ion in various tissues involved in iron metabolism. The best study of this process is the transfer of iron(III) ion from transferrin to the immature erythrocytes. As a model reaction of this process, Bates et al.(1-3) have studied the kinetics of iron(III) ion exchange between a number of chelating agents and transferrin. In addition to a series of the studies of Bates, the present authors have investigated the reaction of apo-transferrin with Fe(III)-NTA by visible and ultraviolet absorption spectroscopy using the stopped-flow technique, and obtained an additional information regarding the reaction sequence of the iron(III) ion binding.

Human serum transferrin(Tf) was prepared from pooled blood which had expired at Central Blood Center. The Cohn fraction IV-4 (4) was eluted from a column of DEAE-Sephadex A50 equilibrated in 0.02 mol/l Tris buffer, pH 8.0 with a concentration gradient of sodium chloride from 0 up to 0.5 mol/l. The procedure to eliminate albumin and α-globulin was essentially the same as the method reported by Inman et al.(5). The Tf fraction so obtained was further chromatographed on QAE-Sephadex A50 column with a concentration gradient of sodium chloride from 0 up to 0.5 mol/l in 0.02 mol/l borate/carbonate buffer, pH 10.0. By this method, hemoglobin could be eliminated completely. The resultant eluent was verified to be pure by polyacrylamide electrophoresis and immunoelectrophoresis (6).

Apo-Tf was prepared by demetallation with EDTA (7). To Tf solution (10^{-4} mol/l), EDTA (10^{-2} mol/l) was added and the pH of the solution was adjusted to 5.0-6.0 with hydrochloric acid. The solution was permitted to stand overnight at 4 °C and dialyzed sufficiently against distilled water before lyophilization.

Iron(III) chloride of a certified reagent grade (Wako Co.Ltd.) was used for the preparation of Fe(III)-NTA complex. NTA (nitrilotriacetic acid) was obtained from Dojindo Laboratories (Kumamoto) and used without further purification. Hydrolysis of Fe(III)-NTA is known to occur so that certain precautions were paid according to Bates et al. (2).

Spectroscopic measurements were performed in 10 m mol/l Tris buffer, pH 7.2 at room temperature using the stopped-flow apparatus (RA-401) and UV/visible absorption spectrometer (SM-401) manufactured by Union Giken Co.Ltd. (Osaka).

When Fe(III)-NTA was added to apo-Tf, the absorption band appeared at 475 nm. Figure 1-(a) shows oscilloscope traces of this process which was detected by the stopped-flow apparatus. The absorbance at 475 nm was saturated after a few seconds of the reaction, while the absorbances at 280 nm and 295 nm continued to increase. On the assumption that the complexation between Fe(III)-NTA and apo-Tf was completed after 3 seconds of the mixing of each solution, the degree of reaction was calculated at different wave lengths. As shown in Figure 1-(b), the absorbances in UV region increased more rapidly than the visible absorbance and further the UV absorbances continued to increase after completion of the visible change. This finding indicates two important features of this reaction; (1) The protein structure changed prior to the formation of the metal complex between Fe(III)-NTA and apo-Tf. (2) The fluctuation of the protein structure continued to occur after completion of the metal complexation so that the ligand exchange reaction mentioned below followed this initial change.

Figure 2 shows second order plots of the reaction of Fe(III)-NTA with apo-Tf. This result indicates that the reaction proceeded via two phases. A similar observation had been reported by Bates et al. (2), who showed a biphasic representation of first order kinetics of the reaction. According to their consideration, the biphasicity results from two-step proceeding of the reaction as shown in equation (1):

$$\text{Fe(III)-NTA} + \text{Tf} \rightleftharpoons \text{NTA-Fe(III)-Tf} \rightleftharpoons \text{NTA} + \text{Fe(III)-Tf} \qquad (1).$$

That is, the first phase was assigned to the specific binding of iron(III) ion to apo-Tf and the second one to the detachment of NTA from the ternary complex, NTA-Fe(III)-Tf. It is, however, well known that Tf specifically interacts with bicarbonate to form a very stable orange complex (8,9). As the ambient concentration of bicarbonate in air-equilibrated solution is $\sim 10^{-3}$ mol/l, the axial ligand of NTA for the ternary complex of NTA-Fe(III)-Tf is capable of exchanging for bicarbonate

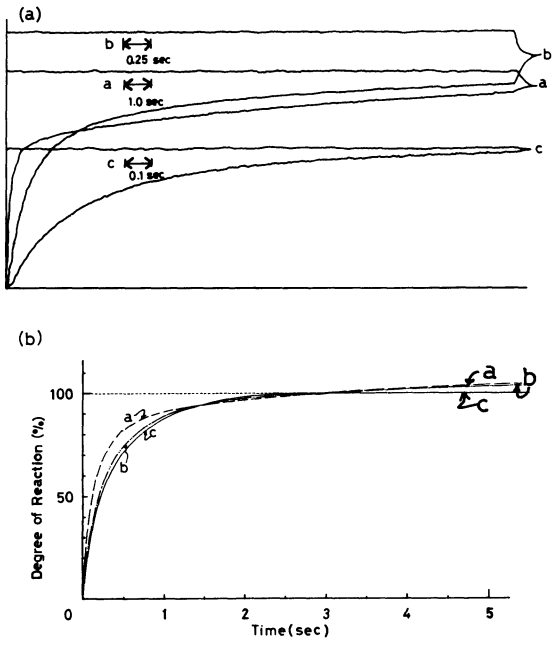

Fig.1: Reaction sequence of the binding of Fe(III)-NTA to apo-Tf. (a) Oscilloscope traces of the stopped-flow measurement; a,280 nm b,295 nm c,475 nm. (b) Time conversion curves determined from Fig.1-(a). $[\text{Fe(III)-NTA}]_o = 6.55 \times 10^{-5}$ mol/l, $[\text{Tf}]_o = 3.25 \times 10^{-5}$ mol/l (per binding site), in 10 m mol/l Tris buffer, pH 7.2, and at 25 °C.

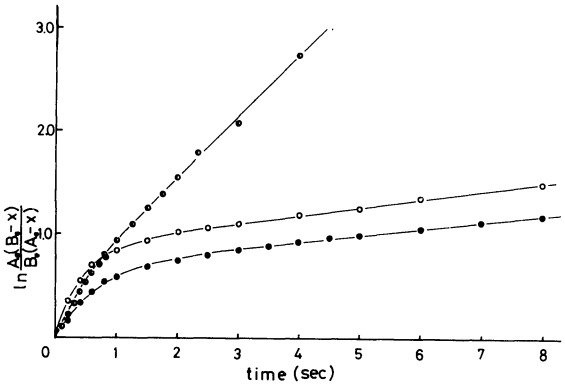

Fig.2: Second order plots of the binding of Fe(III)-NTA to apo-Tf: Scanning wave lengths; -◐- 475 nm, -○- 295 nm, -●- 280 nm. $[Fe(III)\text{-}NTA]_o$=4.14 x 10^{-5} mol/l (Bo), $[Tf]_o$=2.07 x 10^{-5} mol/l (Ao).

dissolved in the solution. On the other hand, the ternary complex of NTA-Fe(III)-Tf is stable in the absence of bicarbonate so that, exactly, the second phase should be assigned to the ligand exchange between NTA and bicarbonate at the axial position of iron(III) ion bound to Tf.

The visible absorption band at 475 nm which is assigned to the initial complex of the reaction of Fe(III)-NTA with apo-Tf gradually changed in a time-scale of 10 minutes, as shown in Figure 3-(b). This change could not be assigned only to the reaction in a coordination sphere of the ternary complex but also to the structural change of the protein (see Fig.3-(a)). The first order plots for the decrease in absorbance at 475 nm were linear and the slopes of the lines were dependent on the bicarbonate and NTA concentrations. That is, the rate of this process was increased with the bicarbonate concentration and decreased with the NTA concentration (see Fig.4). It is thus assumed that this process is the ligand exchange between NTA and bicarbonate that was assigned to the second step in equation (1) by Bates et al.

The present authors consider according to the findings mentioned above that the ligand exchange reaction in the second step in equation(1) proceeds much more slowly than the second phase in Figure 2. Then, two possibilities might be entertained for the explanation of the biphasicity. The first is that there is some difference in the ability of iron binding between the two binding sites of Tf. The second is that there is the difference in reactivity between two or more forms in aqueous solution of Fe(III)-NTA. Although Bates et al. described (2) that heterogeneity of Fe(III)-NTA does not appear to be a significant factor in the characteristics of the reaction with Tf, this possibility must be examined more carefully.

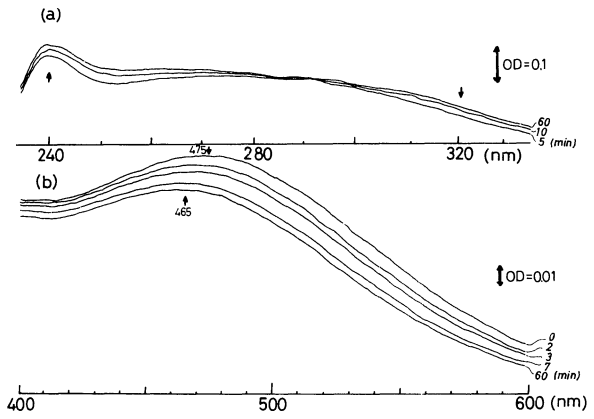

Fig.3: Spectral changes with time in UV and visible regions observed at a later stage of the reaction of Fe(III)-NTA with apo-Tf. $[Fe(III)\text{-}NTA]_o$=7.51 x 10^{-5} mol/l, $[Tf]_o$=13.5 x 10^{-5} mol/l, $[HCO_3^-]_{added}$=6 x 10^{-4} mol/l. The numbers on the spectra indicate the time period in minute after mixing Fe(III)-NTA and apo-Tf.

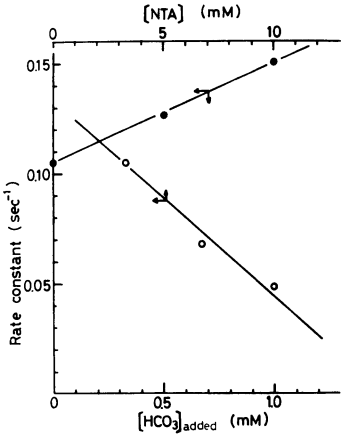

Fig.4: Effect of bicarbonate and NTA concentrations on the change of absorbance at 475 nm at a later stage of the reaction. The concentration of bicarbonate dissolved in air-equilibrated buffer is $\sim 10^{-3}$ mol/l and the concentrations of bicarbonate shown in this figure are the additional ones to the ambient concentration of bicarbonate. Other reaction conditions are the same as that of Fig.3.

We gratefully acknowledge the usefull discussion of Prof.K.Satake (Science University of Tokyo) and the supplying of human transferrin from Mr.O.Nakagawa (Central Blood Center).

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(Received September 17, 1979)