Preparation and characterization of trypsin-nicked ovotransferrin

Hiroshi Ikeda, Yoshiaki Nabuchi, Katsuyoshi Nakazato, Yuichi Tanaka and Kazuo Satake*

Department of Chemistry, Faculty of Science, Science University of Tokyo, 1-3, Kagurazaka, Shinjuku-ku, Tokyo 162, Japan

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The N- and C-domains isolated from ovotransferrin (Tf) with trypsin could be separated from each other and from intact Tf by HPLC with a TSK-GEL G-3000SWG-0.1% SDS system. The analytical method revealed that Fe (III)-saturated Tf (Fe_2Tf) of 77 kDa was hydrolyzed by trypsin preferentially at the portion connecting both domains. The main product was a nicked Fe_2Tf, in which the two fragmented domains of 35 kDa each were still bound together non-covalently and showed a notable cooperativity on their denaturation.

Transferrin *Limited proteolysis* *Denaturation* *Cooperativity*

1. INTRODUCTION

Transferrin, the protein mediating iron transport in vertebrates and iron uptake by their cells [1], is composed of a single peptide chain of 77 kDa. The chain is folded up into two homologous Fe(III)-binding domains, each of which corresponds approximately to the N- and C-terminal halves of the molecule [2]. The functional difference or cooperativity between the two domains, if present, may play an important role in iron metabolism [3]. To elucidate these problems, there have been many studies on the isolation of each domain by the limited proteolysis of various forms of transferrin including ovo- and lactotransferrins. In these studies Fe_2Tf has been assumed to be resistant to trypsin [4,5].

This paper describes that Fe_2Tf can be hydrolyzed with trypsin to a novel derivative with Fe(III)-binding activity, 'nicked Tf', in which the fragmented two domains are bound together non-covalently and the cooperativity between them is still observed on GuHCl and thermal denaturation.

* To whom correspondence should be addressed

Abbreviations: HPLC, high-performance liquid chromatography; Tf, ovotransferrin; Fe_2Tf, Fe(III)-saturated Tf; nicked Fe_2Tf, Fe(III)-saturated nicked Tf; GuHCl, guanidine hydrochloride

2. MATERIALS AND METHODS

2.1 *Transferrin and trypsin modifications*

Apo-Tf, free from trypsin inhibitors [6,7], was prepared in a good yield, (7.3 g from 1 l of homogenized hen egg white) according to Yamamura et al. [8]. The concentration was determined spectrophotometrically, using $A_{280}^{1\%} = 11.3$ [9].

The N- and C-domains were isolated as their Fe(III) complexes according to the methods of Williams, the former from Tf whose N-domain had been preferentially complexed with Fe(III) by digesting the remaining apo-portion with trypsin [5], and the latter from Fe_2Tf by the selective digestion of the less stable N-domain with subtilisin [10].

Nicked Tf was obtained as follows: to prepared Fe_2Tf, 1.7 ml of Fe(III)-niltrilotriacetate complex solution from 20 mM Fe(III) nitrate and 40 mM nitrilotriacetic acid trisodium salt in water [11] was

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added to 1 g apo-Tf dissolved in 20 ml of 0.1 M Tris-HCl buffer containing 5 mM sodium bicarbonate and 10 mM calcium chloride (pH 8.0). To this salmon-pink solution ($\lambda_{max} = 465$ nm), after standing for ½ h at room temperature [12], was added bovine pancreatic trypsin (2 × recrystallized, Sigma) to 1 mg/ml [trypsin/Tf = 1/50 (w/w)] and the mixture was incubated at 45°C for 5 h. The solution still retaining the characteristic color was chilled in an ice-bath and then passed over a column of Sephadex G-100 (Ø 5.5 × 91 cm), using 1 mM ammonium bicarbonate as the solvent. Two colored fractions, 77 and 35 kDa, were dialyzed separately against 0.1 M sodium citrate-hydrochloride buffer (pH 4.7) to remove Fe(III) from the complex [13], then against distilled water, and finally lyophilized, to give nicked Tf and fragmented domain in apo-type, respectively.

2.2 *Analytical methods*

HPLC for the analysis of these Tf components was carried out on TSK-GEL G-3000SWG (a porus silica, Toyo Soda) [14] using a Hitachi 638-30 liquid chromatograph. The sample (150 or 200 μl), after being treated with 5% boiling SDS, was injected and eluted with 50 mM sodium phosphate buffer containing 0.1% SDS (pH 6.5) at a flow rate of 5 ml/min.

The GuHCl denaturation of Fe(III)-Tf complex was achieved in 50 mM Tris-HCl buffer containing 2 M GuHCl (pH 8.0) at 37°C and the decrement of the absorbance at λ_{max}, A_{465}, was recorded in a Hitachi 220A spectrophotometer to measure the time course of dentaturation.

The spectrophotometric analysis of thermal denaturation for Fe(III)-Tf complex was performed in 0.1 M sodium bicarbonate (pH 8.1) by the method of Ikeda et al. [15]. Thus, the A_{465} of sample solution, whose temperature was increased programmatically at a heating rate of 1.5°C/min, was automatically recorded, using a Hitachi 220A spectrophotometer equipped with a thermoelectric cell holder, a temperature programmer (KPC-6) and a temperature controller (SPR-7). Under these conditions, the turbidity due to the heat-coagulated protein was minimized as reported [15].

2.3. *Chemicals*

All chemicals were of the best purity available.

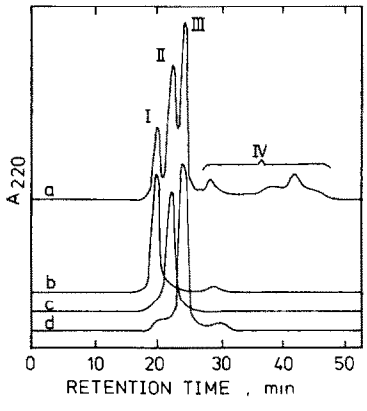

Fig.1. HPLC elution profiles of Tfs. (a) Fe_2Tf treated with trypsin at 45°C for 3 h, (b) intact Tf, (c) fragmented N-domain, (d) fragmented C-domain.

3. RESULTS AND DISCUSSION

3.1. *The chromatographic system for the separation of intact Tf and the two fragmented domains*

The N- and C-domains of Tf are similar in molecular mass (35 kDa) [5,10], so they were eluted very closely from a column of porus silica gel by the usual method [14]: elution of mercaptoethanol-treated sample with a buffer containing SDS and mercaptoethanol. By omitting the procedures for reduction of disulfide linkages, however, the two domains could be easily separated from each other, as shown in fig.1. Any difference between their molecular shapes retained by internal disulfide linkage should result in such a clear separation.

3.2. *The tryptic hydrolysis of Fe_2Tf*

It has been well established by Azari and Feeney [4] that the Fe(III)-binding ability of Fe_2Tf is scarcely damaged by trypsin even after a prolonged incubation at 37°C, differing from that of apo-Tf which was rapidly lost under the same conditions. However, chromatographic analysis of the incubation mixture of Fe_2Tf with trypsin revealed that some portion of Fe_2 Tf was split into two domains, although the original Fe(III)-binding ability was fully retained.

Under the action of trypsin stabilized with Ca^{2+} [16] at 45°C, the limited proteolysis was much more notable, as shown in fig.1a. The main peaks (I–III) corresponded to intact Tf, N- and C-

domains, respectively, and small peaks (IV) to lower peptides. The last components will be derived from the portion connecting the two domains and from the domains further degraded.

The time course of trypsin hydrolysis as summarized in fig.2 indicates that the portion connecting the two domains in Fe_2Tf is rapidly split and the fragmented N-domain is less stable than its partner against the proteolysis at 45°C. The results also show that the loss of Fe(III)-binding ability of Tf during trypsin treatment was mainly due to the degradation of the N-domain.

3.3. *The isolation of nicked Tf*

The trypsin hydrolysate, having no residual intact Tf, was applied to a column of Sephadex G-100 to remove lower peptides. The high molecular mass Fe(III)-Tf complex in the hydrolysate, as shown in fig.3, was found to be resolved into two components of 77 and 35 kDa. HPLC analysis of each component revealed the former to be a 1:1 mixture of N- and C-domains with the latter being the C-domain (in fig.4). These results indicate that trypsin hydrolysis of Fe_2Tf gave an aggregate, in which N- and C-domains were still bound to each other non-covalently (nicked Tf) and then the C-domain fraction was liberated from the nicked Fe_2Tf by preferential degradation of the N-portion.

3.4. *The denaturation of intact and nicked Tf*

The isolated N- and C-domains showed a marked difference in their susceptibility to denaturant as well as to trypsin. On incubation with GuHCl, the isolated N-domain lost Fe(III)-binding ability very rapidly compared to its partner. Under the same conditions, however, the two domains of intact Fe_2Tf denatured simultaneously with a slow rate close to that of the stable C-domain, indicating that interaction between the N- and C-domains stabilized the N-portion of intact Fe_2Tf. Similar results were also observed on nicked

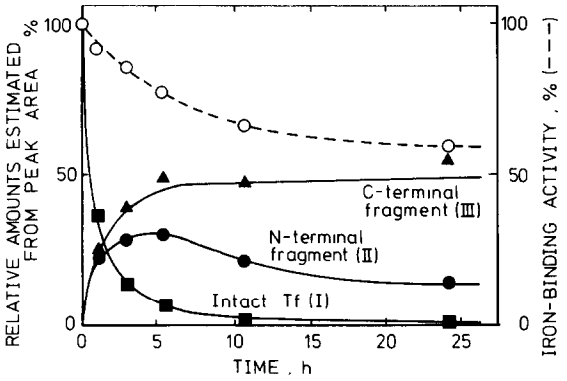

Fig.2. Trypsin digestion of Fe_2Tf at 45°C.

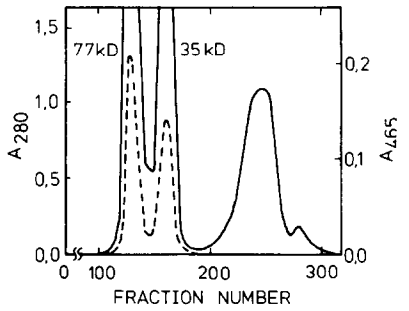

Fig.3. Gel-filtration pattern of Fe_2Tf treated with trypsin at 45°C for 5 h. 9-ml fractions were collected. (——) A_{280}, (– – –) A_{465}.

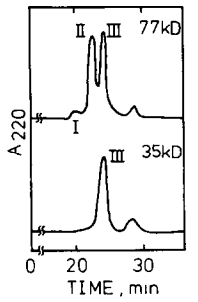

Fig.4. HPLC elution profiles of the 77- and 35-kDa components shown in fig.3.

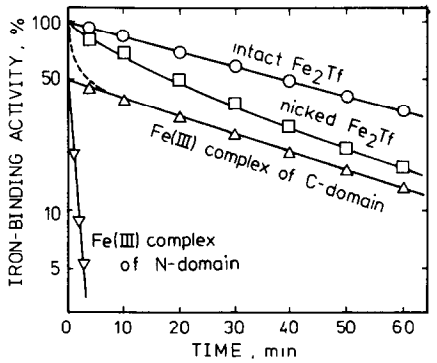

Fig.5. 2 M GuHCl denaturation of Fe-Tf complexes. (——) Curve calculated as a simple sum of a 1:1 mixture of fragmented N- and C-domains.

Fe_2Tf. As shown in fig.5., the time course of denaturation of nicked Fe_2Tf was rather close to that of the intact one and far from the simple sum of Fe(III) complexes of the isolated N- and C-domains.

The stabilization of the N-domain in nicked and intact Fe_2Tf was also indicated on thermal denaturation. As illustrated in fig.6, the 50% denaturation temperature on the N-domain increased from 69.7°C for the isolated one, to 73.0°C for nicked Tf, and to 77.9°C for intact Tf, respectively.

4. DISCUSSION

The results above clearly indicate that Fe_2Tf is hydrolyzed by trypsin preferentially at the connecting portion between the N- and C-domains, provided that the proteinase inhibitors present in egg white [6,7] are completely removed from the Tf preparation by CM- and DEAE-chromatography [8] and trypsin is stabilized with Ca^{2+} [16]. The product of the limited proteolysis is a nicked Fe_2Tf in which the two split domains of 35 kDa each are still bound non-covalently. On prolonged incubation with trypsin, however, the N-domain is gradually degraded, liberating the more stable C-domain. With the use of another proteinase, subtilisin,, Williams [10] has succeeded in isolating the C-domain from Fe_2Tf. The C-domain may also be produced through the initial formation of nicked Fe_2Tf in this preferential proteolysis

There are many reports on the limited cleavage

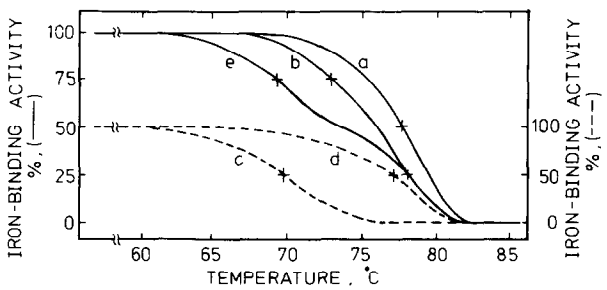

Fig.6. The thermal denaturation curves of Fe-Tf complexes. (a) Intact Tf, (b) nicked Tf, (c) fragmented N-domain, (d) fragmented C-domain, (e) mixture of fragmented N- and C-domain. The denaturation temperature (T_m, the temperature at the point of half denaturation) of each sample is indicated by +.

of the intact proteins to the nicked products: proteinase-catalyzed conversion of ovalbumin to plakalbumin [17,18], subtilisin-modified RNase A [19], proteinase nicked-thioredoxin [20], oxidative sulfitolysis of immunoglobulin G to 'Venoglobulin S' [21,22], and trypsin hydrolysis of diphtheria toxin followed by the reductive cleavage of the disulfide linkage between the fragmented domains [23]. These nicked derivatives have afforded valuable information on the function and structure of these proteins, which is not available from studies on the intact proteins. The results here strongly suggest that the N-domain of intact Fe_2Tf is cooperatively stabilized with the partner domain and that the effect still remains in nicked Fe_2Tf. The nicked Tf will be a promising material for studies on the cooperatively between the two domains, not only in structure stabilization but also in the binding functions with ligands, Fe(III) and CO_3^{2-}, and with the Tf-receptor on the cell surface.

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