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In vitro observation of interactions of iron and transferrin by capillary isoelectric focusing with a concentration gradient imaging detection system

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ABSTRACT

A capillary isoelectric focusing–concentration gradient imaging detector system was used for *in vitro* observation of dynamics of interaction between iron and bovine transferrin. Transferrin was first focused inside the capillary by isoelectric focusing. A plug of ferric ion was then introduced into the capillary. The iron-free and the iron-complexed transferrins have different isoelectric points, hence they were focused at different positions inside the capillary. Concentration changes of different isoforms of iron-free and iron-complexed transferrins were monitored in an on-line fashion during the interaction by the imaging detector. One advantage of using this detector for studying the protein interaction is that the 633 nm wavelength probe beam used in the detector does not pump energy into the reaction system, which will interfere with the reaction. The results show that iron-binding and dissociation rates of different isoforms are different. This is the first report that the reaction speed of different isoforms of a protein can be observed in an on-line fashion.

INTRODUCTION

Capillary electrophoresis (CE) has become an important separation method in analytical chemistry and biochemistry. Separation and detection of different components in small amounts of sample (pl–nl in volume) can be achieved in a few minutes [1]. Because of its high separation efficiency and speed, CE is also a promising tool for the study of interactions between biologically active molecules, such as in the characterization of enzyme–antibody conjugates [2], the separation of antibody–antigen complexes [3], and the study of protein–drug binding [4]. In these works, studies of the interactions of those molecules under different concentration ratios were achieved by capillary zone electrophoresis (CZE) which separated the conjugates and the complexes from unreacted molecules based on their differences in electrophoretic mobility [2–4]. In this way, binding parameters between molecules could be determined. However, until now there is no method in which one can observe the dynamics of the interactions between the different isoforms of different molecules in an on-line fashion.

In order to observe the interaction dynamics, two conditions are required. First, a special "container" is needed, in which the interaction takes place. In the "container", different isoforms of a protein, and the reacted and unreacted species can be distinguished. An on-line detector is also necessary to monitor the concentration changes of all species. Capillary isoelectric focusing (cIEF) [5] is a suitable "container" for this purpose. In cIEF, all proteins inside the capillary are focused at the positions where their isoelectric points (p*I*) are equivalent to the pH. It is a static situation. It has a resolution of 0.02 pH units [5]. It is a powerful method for resolving different isoforms of protein samples. Also,

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if the reacted species and unreacted species have different p*I* values, they will be focused at different positions inside the capillary. However, in order to observe changes of concentrations of all these species inside the capillary during the interaction process, protein zones need to be detected by an on-line imaging detector. All present commercial CE detectors are not suitable for the purpose.

In our laboratory, a concentration gradient imaging detector based on Schlieren optics [6] and a UV–Vis absorption imaging detector [7] were developed for cIEF detection. For observation of dynamics of protein reaction, concentration gradient imaging detection had advantages over other optical and spectroscopic detectors. A low-power, long-wavelength He–Ne laser (633 nm) was employed in the detector. This eliminated the possibility of pumping energy into the reaction system, which might disturb the reaction. Thus, the cIEF–concentration gradient imaging detector system [6] is an ideal instrument for the observation of the dynamics of interaction between biological molecules. In this report, the feasibility of using the cIEF–concentration gradient imaging detector system for observing the dynamics of interaction between biological molecules will be shown by observing the interactions between bovine transferrin and iron.

EXPERIMENTAL

Instrumental

The cIEF–concentration gradient imaging detector system used has been described in detail in our previous paper [6]. It consists of a capillary cartridge holding a 100 μm I.D., 4 cm long square glass capillary (Dynamics, Rockaway, NJ, USA), a low-power He–Ne laser (Model 1103P, Uniphase, Manteca, CA, USA), and a 1024 pixel CCD sensor (S3903-1024Q, Hamamatsu, Hamamatsu City, Japan). The capillary inner wall was coated with non-cross-linked acrylamide to eliminate electroosmosis by the reported method [5].

Reagents

All solutions used in the experiment were prepared using deionized water. All chemicals were reagents grade. Solutions of 10 m*M* H_3PO_4 and 20 m*M* NaOH were used as anolyte and catholyte, respectively. Proteins were purchased from Sigma, St. Louis, MO, USA. Iron-free and iron-saturated bovine transferrin were used in the experiment. Protein samples were mixed with the carrier ampholytes (Pharmalyte pH 5–8, Sigma) solution to a final concentration of 2% ampholytes. Protein concentrations were 0.5 mg/ml. Iron(III) solution of $5 \cdot 10^{-4}$ *M* was prepared in 10 m*M* H_3PO_4.

Procedures

The protein solution was introduced into the capillary by pressure. Two buffer reservoirs at the two ends of the capillary were filled with anolyte and catholyte, respectively. Then a 3.5 kV dc voltage was applied to the two ends of the capillary. The current which passed through the capillary was monitored to follow the focusing process and interaction dynamics between iron and transferrin. The current dropped from about 30 to 3 μA in two min before stabilizing. After 2 min of focusing, the anolyte in the reservoir at the anodic end of the capillary was replaced by the iron solution for 20 s, and the reservoir was filled again with the anolyte. In this way, a plug of iron solution was introduced into the capillary. Protein zones inside the capillary were monitored on-line by the concentration gradient imaging detector. All experiments were done in duplicate or triplicate to ensure reproducibility.

RESULTS AND DISCUSSION

In the experiment, carrier ampholytes having pH values ranging from 5 to 8 were used, giving good resolution for isoforms of transferrin. After application of the dc voltage, a pH gradient from pH 5 to pH 8 was established along the capillary, and proteins having p*I* values in this range were focused inside the capillary. Proteins are focused where the pH equals their p*I* values. All these protein zones inside the capillary can be detected by the concentration gradient imaging detector. The signal profile of the detected image is proportional to the second derivative of the protein sample concentration [6].

First, iron-free and iron-saturated transferrin

were separated by the cIEF instrument. As shown in Fig. 1a and c, at least four isoforms can be distinguished for the iron-free transferrin by this method. The isoforms are located in the pH range of 5.30–5.60. Each isoform may bind iron at several binding sites [8], so there can be many iron-complexed isoforms. The focused pattern for iron-saturated transferrin is rather complicated as shown in Fig. 1a. The iron-transferrin complexes have different p*I* values from those of iron-free transferrin [8]. As shown in Fig. 1a, they distribute in the pH range of 5.15–5.30.

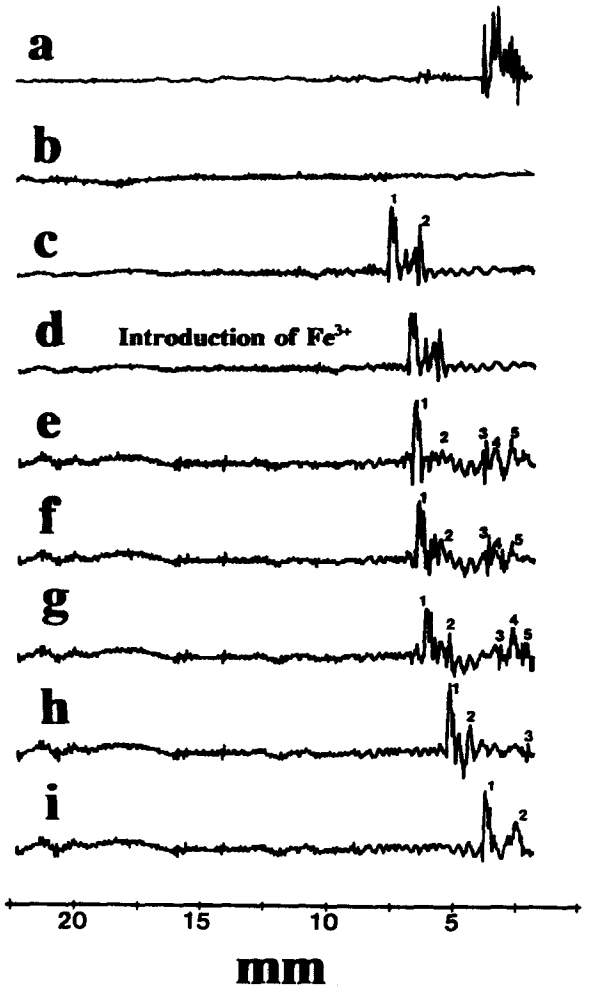

Fig. 1. (a) Focused pattern of iron-saturated bovine transferrin after 2 min focusing. Bovine transferrin of 0.5 mg/ml concentration are focused by the cIEF instrument, and the focused patterns were detected by the imaging detector (b, c). After 2 min focusing, a plug of ferric ion was introduced into the capillary, and the changes in the focused pattern were monitored (d–i). (b) Image of iron-free bovine transferrin after 3 s of focusing, and (c) after 2 min of focusing. (d) Focused pattern of iron-free transferrin 20 s after the introduction of the ferric ion plug, (e) 0.5 min, (f) 2 min, (g) 4 min, (h) 8 min, and (i) 12 min. Peaks 1 and 2 are iron-free forms of transferrin, peaks 3–5 correspond to iron–transferrin complexes.

The focused pattern of the proteins became stable after 2 min. If ferric ions are introduced into the capillary at this point, they should be bounded by the transferrin, and the position of each isoform of the protein inside the capillary should change upon binding [8]. This position change could be observed on-line by the imaging detector. Thus, the dynamics of the interaction between iron and transferrin could be monitored. The iron–transferrin complexes were formed with the participation of anions [9]. Furthermore, the existence of the carrier ampholytes facilities their formation [8]. In our experiment, the H_3PO_4 solution of ferric ion effectively prevented the ion from precipitating at high pH conditions due to $Fe(HPO_4)^+$ formation.

There is a problem with the introduction of the ferric solutions into the capillary. Introducing ions other than H^+ to the anodic end of the capillary will destroy the pH gradient along the capillary, and cause the focused proteins to mobilize toward this end of the capillary [5]. If the concentration of the ferric ion is too high, all focused proteins will be mobilized out of the capillary in a short time, which makes it difficult to observe the interaction between ferric ion and the protein. If the concentration is too low, the concentrations of iron–transferrin complexes will be too small to be detected by the detection system. These difficulties can be solved by introducing a ion plug into the capillary. We found in experiments that when a K^+ plug ($2 \cdot 10^{-3} M$) was introduced into the anodic end of the capillary after all proteins were focused, mobilization caused by the plug was slow. All focused zones could stay in the 4-cm long capillary for more than 10 min. Mobilization of the pH gradient caused by a low concentration Fe^{3+} plug is also slow. The mobilization speed can be monitored by measuring the current passing through the capillary. As illustrated in Fig. 2, after application of the dc voltage, the current stabilized in about 2 min. This shows that a stable pH gradient is established in the capillary. When a

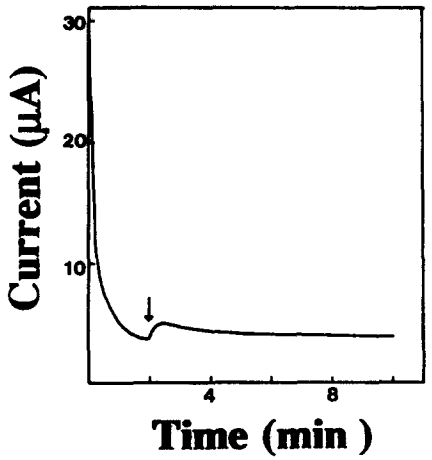

Fig. 2. The current passing through the capillary during the focusing process of the protein and the interaction between iron and transferrin. The time the ferric ion plug is added was marked by the arrow.

plug of $5 \cdot 10^{-4}$ *M* ferric ion was introduced, there was an increase in current passing through the capillary. However, because of the short length of the plug, the plug moves through the capillary. The current almost becomes stable in 1 min, and just increases very slowly, which means that after 1 min the mobilization of the pH gradient inside the capillary is slow.

Peaks in Fig. 1c represent different isoforms of iron-free transferrin, and each isoform may exist in iron-free and iron-complexed molecular forms. In addition, because each isoform has several iron binding sites, every iron-free isoform can create several different iron-complexed isoforms. The major advantage of using the cIEF-imaging detector system to observe the interaction between iron and transferrin is that the association and dissociation rates of different isoforms can be monitored in an on-line fashion. The association constants for different iron-complexed isoforms can be estimated since the peak height in the detected image is approximately proportional to the concentration of the protein.

Fig. 1c shows two main peaks (peaks 1 and 2) for iron-free transferrin. Immediately after the introduction of the ferric ion plug, as shown in Fig. 1d, all focused zones are mobilized quickly toward the anodic end of the capillary by the ferric ion. This corresponds to the current increase in Fig. 2 marked by the arrow. However, the focused pattern of the iron-free transferrin does not change. The quick mobilization only lasts for about 1 min. After 1 min, the mobilization is very slow in 10 min as shown in Fig. 1e–i, which allows observation of the interaction between ferric ion and transferrin.

The focused pattern begins to change 1 min after the introduction of ferric ion due to the interaction between the ion and the protein (Fig. 1e). After the addition of ferric ion, peak 2 quickly disappears. However, several new peaks (peaks 3–5) appear in the positions of iron–transferrin complexes as shown in Fig. 1a. Peak 1, which corresponds to an isoform of iron-free transferrin, also becomes lower slowly as shown by Fig. 1e–g. These results show that peaks 3, 4, and 5, which are iron–transferrin complexes are mainly formed from peak 2. Under these conditions, after the ferric ion zone passes through the capillary, peaks 3–5 become lower as shown in Fig. 1f–h. This is because under these conditions any ferric ions dissociated from the complexes are immediately rejected from the capillary by the applied high dc voltage applied at the two ends of the capillary, the dissociated transferrin returns to its original position. This is observed in Fig. 1f–i, where peaks 1 and 2 slowly become higher again. Another interesting phenomenon is the formation and dissociation speed of peaks 3–5. The formation speed of peak 3 is the fastest; however, it also disappears in the fastest rate (Fig. 1e–h). The formation and dissociation speeds of peaks 4 and 5 are both slower than that of peak 3. After 12 minutes, most of iron–transferrin complexes are dissociated, and the peak shapes of the iron-free transferrin are almost restored to their original forms (Fig. 1c and i). By this instrument, much information can be obtained for the interaction between iron and transferrin during the short time interaction. This achievement is impossible for a conventional cIEF instrument with a single point on-column detector using mobilization processes [5].

The above results show that the cIEF–concentration gradient imaging detector system is a powerful tool for the study of the interactions between biochemical molecules. Future work will concentrate on the study of interactions between monoclonal antibodies and antigens,

and the evaluation of the association constants of their conjugates.

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