Effect of synthetic carrier ampholytes on saturation of human serum transferrin

Arduino ORATORE,* Anna M. D'ALESSANDRO and Gabriele D'ANDREA

Dipartimento di Scienze e Tecnologie Biomediche e Biometria, Università degli Studi dell'Aquila degli Abruzzi, Collemaggio, 67100 L'Aquila, Italy

We have investigated the effect in solution of synthetic carrier ampholytes on the saturation of human serum transferrin. By spectrophotometric titrations of human serum transferrin with various Fe^{3+} -carrier ampholyte solutions, we demonstrated that under these conditions carrier ampholytes behave as typical chelators, their binding curves being very similar to that obtained with disodium nitrilotriacetate. On performing titration experiments at three different pH values, carrier ampholytes act like nitrilotriacetate at pH 7.5, but the former are more effective iron donors at pH 8.4 and worse iron donors at pH 5.2. Spectrophotometric titrations of isolated C-terminal and N-terminal fragments obtained from human serum transferrin by thermolysin cleavage show no differences between them, and no differences with respect to the whole protein except that they contain half the number of binding sites. In order to determine a site-specificity of iron in the presence of ampholytes, the classical urea/polyacrylamide-gel-electrophoresis technique was adopted. Under saturating conditions carrier ampholyte solutions act mostly on the C-terminal site, whereas desaturating agents remove iron preferentially from the N-terminal site. Our findings support the hypothesis that Ampholine may chelate Fe^{3+} as well as many other compounds.

INTRODUCTION

Human serum transferrin (HST) is a protein involved in iron exchange between tissues. It is a bilobal glycoprotein with two separated and independent iron-binding sites located in the N-terminal and C-terminal half-domains. In the presence of a synergistic anion, which physiologically is HCO_3^- , one transferrin molecule binds very tightly two Fe^{3+} ions, with an association constant in the order of 10^{22} M^{-1} (Aisen & Listowsky, 1980; Chasteen, 1983).

Detailed studies report that transferrin reacts very poorly with non-chelated Fe^{3+} salt solutions at neutral pH (Bates & Schlabach, 1973). By contrast, iron is readily bound or delivered to transferrin in the presence of chelating agents such as nitrilotriacetate (NTA), EDTA and citrate to form chelate- Fe^{3+} -protein intermediates. Furthermore, the nature of the Fe^{3+} complex confers a site-specificity to iron, which prefers to occupy at first the C-terminal site (e.g. Fe^{3+} -NTA) or the N-terminal site (Fe^{3+} -citrate, Fe^{3+} -oxalate) (Aisen *et al.*, 1978).

Previously, iron distribution in transferrin samples has been analysed by an isoelectric focusing-carrier ampholytes technique: by this means three iron-containing species can be detected, corresponding to the apo, monoferric and diferric forms (Hovanessian & Awdeh, 1976). Moreover, isoelectric focusing is widely used for the determination of transferrin genetic variants in forensic investigations (Constans *et al.*, 1980). Nevertheless many authors have reported evidence of an interaction between iron and carrier ampholytes that altered the expected distribution (Righetti & Drysdale, 1976; Constans *et al.*, 1980). During our previous study we

analysed HST patterns by isoelectric focusing in the presence of free carrier ampholytes (Ampholine) or immobilized ampholytes (Immobiline), and we demonstrated that only free ampholytes are able to change the initial distribution of iron, leading to artifactual results (D'Alessandro *et al.*, 1988).

On the basis of this evidence we have investigated this effect in solution and we have hypothesized a mediated chelating action by carrier ampholytes. For this purpose we have performed, under different experimental conditions, spectrophotometric titrations of apo-transferrin or its N-terminal or C-terminal fragments alone with Fe^{3+} plus ampholytes in comparison with the effective and well-known chelator NTA.

In order to determine a site-specificity of iron in the presence of ampholytes the classical urea/polyacrylamide-gel-electrophoresis technique has been adopted (Makey & Seal, 1976).

MATERIALS AND METHODS

Chemicals

All chemicals were analytical-grade reagents. Buffers and acrylamide solutions for electrophoresis were passed through a column of chelating resin (Sigma Chemical Co., St. Louis, MO, U.S.A.) to remove extraneous metal ions.

In our experiments synthetic carrier ampholytes Ampholine (pH 5–7 range) and Immobiline (pK 3.6, 4.6, 6.2, 7.0 and 9.3) (LKB, Bromma, Sweden) were used.

Acrylamide, bisacrylamide, ammonium persulphate, *NNN'*-tetramethylethylenediamine and Coomassie Brilliant Blue R-250 were purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A.

Abbreviations used: HST, human serum transferrin; NTA, nitrilotriacetate.

* To whom correspondence should be addressed.

Urea was obtained from Sigma Chemical Co. To eliminate carbamate, urea solution was deionized by mixed-bed ion-exchange resin (Bio-Rad AG 50 1-X8).

Preparation of chelate complexes

All ferric chelate complexes were prepared by using FeCl_3 as the iron source. Standard Fe^{3+} -NTA chelate complex was freshly prepared by mixing equal volumes of FeCl_3 and NTA solution in the molar ratio 1:2, and adjusting the pH of the complex to 6.0 by dropwise addition, under vigorous stirring, of 0.1 M-NaOH; the final Fe^{3+} concentration was 1.25 mM.

Fe^{3+} -Ampholine solution was prepared by mixing equal volumes of Ampholine (pH 5-7) at the following final concentrations: 2 %, 10 % and 20 % (v/v) and Fe^{3+} at the final concentration of 1.25 mM.

Immobiline solution (0.2 M) was prepared as previously reported (D'Alessandro *et al.*, 1988) and mixed with FeCl_3 (1.25 mM).

Protein preparation

HST was purchased from Behring (Scoppito, L'Aquila, Italy), and after SDS/polyacrylamide-gel-electrophoretic analysis for purity assay was used without further purification. Apo-transferrin was prepared as previously reported (D'Alessandro *et al.*, 1988). Diferric HST (2Fe-HST) was prepared by adding to apo-transferrin suitable amounts of Fe^{3+} -NTA, calculated by parallel titration experiments (see Fig. 1). The N-terminal fragment of HST was prepared by thermolysin digestion by the procedure of Lineback-Zins & Brew (1980). The C-terminal fragment was obtained by thermolysin cleavage of the protein, exploiting the resistance of the iron-saturated transferrin binding site to denaturation and proteolysis, by the procedure of Zak & Aisen (1985).

All protein preparations were in 0.1 M-Tris/HCl containing 10 mM- NaHCO_3 , pH according to the experiment.

Titration experiments

Titration experiments were performed by adding to 1 ml of apo-transferrin (61.2 μM) or C-terminal fragment (122.5 μM) or N-terminal fragment (122.5 μM) increasing amounts of 1.25 mM-ferric chelate complex. After mixing, the absorbance was immediately read and corrected for dilution. pH was checked carefully and did not change during the titration experiments. Absorbance was monitored at 470 nm.

Urea/polyacrylamide-gel electrophoresis

The urea/polyacrylamide-gel electrophoresis was performed as described by Makey & Seal (1976) with slight modifications. Briefly, gels were prepared in 89 mM-Tris/borate buffer, pH 8.4, containing 6 M-urea and 6.5 % acrylamide/bisacrylamide. Stock solutions of 28.4 % acrylamide/1.6 % bisacrylamide and 2 M-Tris/borate buffer, pH 8.4, were used. Samples containing about 50 μg of transferrin in 0.1 M-Tris/HCl buffer, pH 7.5, containing 10 mM- NaHCO_3 and 20 % (v/v) glycerol were applied to each slot of the vertical gel slab, which was 1.5 mm thick. The electrophoresis was run at 100 V for 20 h. Gels were stained with Coomassie Brilliant Blue R-250.

RESULTS AND DISCUSSION

When diferric HST was incubated with Ampholine (pH 5-7) the absorbance of the solution of 470 nm decreased by about 30 % within 5 min, clearly indicating iron release from transferrin (results not shown). The same mixture was also analysed by urea/polyacrylamide-gel electrophoresis, which confirmed that the diferric protein was converted partly into the apo form and mostly into the C-terminal monoferric form (FeC-HST). On the other hand, when iron was added to apo-transferrin in the presence of Ampholine, an absorbance increase was found, due to the conversion from the apo form into the iron-containing protein. Furthermore, kinetic measurements made with a stopped-flow apparatus showed a rapid saturation (in the order of a few milliseconds) of the apo-transferrin by Fe^{3+} -Ampholine (2 %, v/v) (results not shown).

On the basis of these data, the iron-binding capacity of HST in the presence of Ampholine was tested by spectrophotometric titration of apo-transferrin with Fe^{3+} -Ampholine (pH 5-7). A curve with a linear response and a clear end point, corresponding to 1:1 molar ratio of Fe^{3+} /binding site, was obtained (Fig. 1). This behaviour is typical of a Fe^{3+} -chelate complex, e.g. Fe^{3+} -NTA, used in our experiments as control. Moreover, as shown in Fig. 1, a dependence on the Ampholine concentration was established, the lowest effective concentration being about 2 % (v/v). Immobiline lacks this ability, showing a flat titration curve.

It is well known that HST binds iron tightly at around pH 7.5, whereas at acidic pH its affinity is lowered significantly (Chasteen & Williams, 1981). Taking into account this phenomenon, we performed spectrophotometric titrations at three different pH values: under otherwise the same experimental conditions, the highest final absorbance value was reached at pH 8.4 (Fig. 2). In particular, it can be pointed out that Ampholine and NTA behave in the same way at pH 7.5; they are more effective at pH 8.4 and less effective at pH 5.2. As far as Ampholines of different pH ranges are concerned, all the commercially available batches tested in our experiments

Fig. 1. Chelating effect of Ampholine

Spectrophotometric titrations of apo-transferrin at pH 7.5 with Fe^{3+} presented as FeCl_3 (○) or Fe^{3+} -NTA (□) or Fe^{3+} -Ampholine (pH 7.5) at different concentrations of Ampholine [2 % (v/v) (■); 10 % (v/v) (▲); 20 % (v/v) (●)] or Fe^{3+} -Immobiline (0.2 M) (▼). All solutions contained 1.25 mM- Fe^{3+} . The arrow indicates the 1:1 Fe^{3+} /binding site ratio.

Fig. 2. Effect of pH on the Ampholine chelation

Spectrophotometric titration of apo-transferrin with Fe^{3+} -NTA ($\circ$, $\triangle$ and $\square$) or Fe^{3+} -Ampholine ($\bullet$, $\blacktriangle$ and $\blacksquare$) at different pH values: pH 7.5 ($\circ$ and $\bullet$); pH 8.4 ($\triangle$ and $\blacktriangle$); pH 5.2 ($\square$ and $\blacksquare$). The starting solutions contained 1.25 mM- Fe^{3+} and 20% (v/v) Ampholine.

Fig. 3. Urea/polyacrylamide-gel electrophoresis of forms of transferrin

Lane 1, apo-transferrin; lane 2, transferrin 30% saturated with Fe^{3+} -Ampholine (pH 5–7); lane 3, diferric transferrin; lane 4, diferric transferrin after incubation with Ampholine (final concentration 2%, v/v).

showed a similar effect, with a strict dependence on the experimental pH value (results not shown).

One more feature of iron donors is their site-specificity: e.g., Fe^{3+} -NTA is known to saturate preferentially the C-terminal site of HST (Aisen *et al.*, 1978). We have investigated this aspect by urea/polyacrylamide-gel electrophoresis, which clearly shows that Ampholine is

Fig. 4. Chelating effect of Ampholine on HST fragments

Spectrophotometric titrations of apo-transferrin ($\circ$ and $\bullet$), apo-C-terminal fragment ($\triangle$ and $\blacktriangle$), apo-N-terminal fragment ($\square$ and $\blacksquare$) at pH 7.5 with Fe^{3+} -NTA ($\circ$, $\triangle$ and $\square$) or Fe^{3+} -Ampholine ($\bullet$, $\blacktriangle$ and $\blacksquare$). Fe^{3+} -NTA and Fe^{3+} -Ampholine concentrations were the same as indicated in Fig. 2 legend.

mostly specific for the C-terminal site when apo-transferrin is saturated, but when diferric transferrin is desaturated in the presence of Ampholine iron is preferentially removed from the N-terminal site (Fig. 3). Spectrophotometric titrations of the isolated C-terminal and N-terminal fragments tested under the same experimental conditions show that both fragments are readily saturated, similarly to the whole HST (Fig. 4).

This behaviour of Ampholine strongly resembles that of many other substances investigated, and we can argue that Ampholine may, in the same way, chelate Fe^{3+} . This hypothesis should be supported by specific kinetic and structural studies, which are not easily achievable because Ampholines are usually supplied as a mixture of different ampholytes with different chemical structures.

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