Binding of various ovotransferrin fragments to chick-embryo red cells

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1. The ability of *N*- and *C*-terminal half-molecule fragments of hen ovotransferrin to interact with chick red blood cells (CERBC) has been studied under conditions that allow binding of the transferrin to transferrin receptors to take place, but not the delivery of iron to the cell. Two kinds of half-molecule fragments were used: (a) those which can associate with one another to give a dimer resembling native transferrin and (b) those which cannot associate in this way because they lack a few amino acid residues from their *C*-terminal ends. 2. Neither *N* nor *C* half-molecules alone can bind to the CERBC, but, when both are present, tight binding occurs. 3. Whether or not the half-molecules can associate with one another makes little difference to receptor binding. 4. Given that one of the half-molecules is iron-saturated, the presence or absence of iron in the contralateral half-molecule again makes little difference to receptor binding.

INTRODUCTION

Transferrins are a group of glycosylated iron-binding proteins of similar amino acid sequence which are widely distributed in various biological fluids of all vertebrates (Feeney & Komatsu, 1966; Aisen & Listowsky, 1980). The protein ($M_r \sim 80000$) has two similar globular domains, each containing a specific binding site for Fe(III) (Aisen & Listowsky, 1980), and it is involved in iron delivery to dividing cells (Brock & Mainon-Fowler, 1983).

In spite of numerous studies describing the properties of the transferrin-receptor system and of the transferrin-cell cycle (Harding *et al.*, 1983; Iacopetta & Morgan, 1983; Klausner *et al.*, 1983a), very little is known about the precise mechanism triggering the internalization of the receptor-transferrin complex or about the specific regions of transferrin and its receptor which interact with each other.

Important information has been recently published elucidating transferrin binding and iron uptake in the ovotransferrin-CERBC system (Keung & Azari, 1982; Brown-Mason & Woodworth, 1984; Ikeda *et al.*, 1985). Brown-Mason & Woodworth (1984) showed that isolated *N* or *C* half-molecule fragments could not bind to CERBC alone, but that, when both *N* and *C* are present, binding and iron delivery occur. Ikeda *et al.* (1985) have shown that, after cleaving the connecting peptide of ovotransferrin with trypsin, the two domains remain non-covalently attached to one other and continue to show co-operative interactions. Brown-Mason *et al.* (1987) have suggested that the ability of mixtures of *N*- and *C*-terminal half-molecules to bind to the transferrin receptor and to deliver iron to the cells depends upon their prior non-covalent association. Recently, Williams & Moreton (1988) have prepared, by partial proteolysis under controlled conditions, novel types of fragments from hen ovotransferrin. These well-defined forms of the *C*- and *N*-fragments lack a few amino acid residues from their *C*-

terminal ends and have also lost their ability to associate with each other. In the present work the ability of these iron-complexing *N* and *C* half-molecules to bind to CERBC has been studied.

MATERIALS AND METHODS

Materials

Sephadex G-25 and Sephadex G-100 were purchased from Pharmacia. $^{59}\text{FeCl}_3$ (100 $\mu\text{Ci}/\text{ml}$ in HCl) and Na^{125}I (carrier-free; 100 mCi/ml in NaOH) were from Amersham. Centricon microconcentrators were obtained from Amicon.

Protein preparation

Ovotransferrin was purified as described by Williams (1968). The ovotransferrin-derived half-molecules were obtained and purified as detailed previously (Williams & Moreton, 1988). Briefly, the *N* and *C* fragments, corresponding to their respective domains in the intact protein, were derived from tryptic cleavage of iron-saturated ovotransferrin in the presence of 0.02 M- CaCl_2 (Brock *et al.*, 1976).

After purification by anion-exchange chromatography they were submitted to a further chymotryptic cleavage, giving two 'uncomplexable' fragments, N_u and C_u . All the protein preparations were homogeneous by conventional SDS/PAGE analysis. In order to avoid any contamination of ovotransferrin iron-free fragments with extraneous iron or other metal ions, all buffers used for protein preparation and the following binding studies were passed through a column of Chelex-100 (Bio-Rad Laboratories).

Ability of fragments to associate

Fragments were tested for their ability to associate by running mixtures of both fragments on a Sephadex G-100 column and comparing their elution volumes with

Abbreviations used: CERBC, chick-embryo red blood cells; EGA, Earle's salts containing 6 mM-glucose, 2.5% (w/v) bovine serum albumin; Fe-NTA, ferric nitrilotriacetate; PAGE, polyacrylamide-gel electrophoresis.

those of the whole protein and of the same fragments alone. No further test was carried out, as extensive studies have been performed in this laboratory and reported elsewhere (Williams & Moreton, 1988).

Radiolabelling procedure

Apo forms of ovotransferrin or fragments were prepared by dialysis against 0.1 M-citrate, pH 4.7, or 0.2 M-potassium phosphate/1 mM-NTA/1 mM-EDTA, pH 6.0, respectively (Brown-Mason & Woodworth, 1984). ^{59}Fe labelling was carried out by a similar procedure (Williams, 1974). In particular, each protein, dissolved in 20 mM- NaHCO_3 , was added to an appropriate amount of ^{59}Fe , as $^{59}\text{FeCl}_3$, which had been previously dried and buffered with 1 M- NH_4HCO_3 . A solution of non-radioactive Fe-NTA, calculated by accurate spectrophotometric titration on a separate protein solution to give full saturation, was then added. The specific activity ranged from 50 to 100 Ci/mol of protein in different preparations. Trace-labelling with ^{125}I was performed essentially as described by Keung & Azari (1982). Briefly, 10 mg of protein was taken up in 200 μl of 0.05 M-potassium phosphate buffer, pH 7.6, to which 300 μCi of Na^{125}I and 10 μl of chloramine-T (10 mg/ml) were added. The reaction was allowed to proceed for 5 min at room temperature with occasional mixing. Then 20 μl of KI (100 mg/ml) were added to dilute the ^{125}I . The reaction mixture was passed through a Sephadex G-25 column (8 ml of resin in the barrel of a 10 ml disposable syringe fitted with a 22-gauge needle and plugged with glass wool) equilibrated with EGA medium. The protein-containing fractions were concentrated, washed, and exchanged into the buffer of choice on a Centricon-10 microconcentrators.

Binding studies

A simplified experimental scheme as described by Brown-Mason & Woodworth (1984) was adopted.

CERBC, collected from 14-day-old chick embryos, were washed three times with EGA and then incubated in a large excess of the same medium at 37 °C for 30 min to eliminate endogenous ovotransferrin. After centrifugation, the procedure was repeated twice. Packed cells were resuspended in a volume of the same buffer solution containing 40 mM- NH_4Cl to give a final cell number ranging from 1.0×10^7 to 2.0×10^7 ml, as determined with a Thoma-Zeiss counting chamber. After that, all binding experiments were carried out in the presence of 20 mM- NH_4Cl to prevent iron release from ovotransferrin (Morgan, 1981; Harding & Stahl, 1983; Klausner *et al.*, 1983b; Rao *et al.*, 1983; Brown-Mason *et al.*, 1987).

Cell aliquots

Portions (0.2 ml) of cells were pipetted into plastic tubes containing 0.1 ml of different concentrations of the labelled protein or of a mixture of the labelled fragment with a 2-fold excess of the non-radioactive contralateral half-molecule. After incubation at 37 °C for 30 min, samples were centrifuged and the radioactivity of the supernatant measured. Calculations were made with respect to a standard curve, and the number of molecules bound per cell plotted against the concentration of free ligand. These curves were corrected for non-specific binding as described by Brown-Mason & Woodworth (1984).

RESULTS

Dimerization of half-molecule fragments

The loss of ability to associate of *N* and *C* fragments after chymotryptic cleavage was confirmed by running mixtures of these fragments with their contralateral completable fragments on Sephadex G-100. Fig. 1 shows that their elution volumes are significantly increased with respect to that of a mixture of both completable fragments, clearly indicating lack of association.

Fig. 1. Sephadex G-100 elution profile after loading 0.5 ml samples containing 5 mg of ovotransferrin (●), 5 mg of *C* fragment (△), 5 mg of *N* fragment (□), 3 mg of *N*₁ fragment and 3 mg of *C*-fragment (○), 3 mg of *N*-fragment and 3 mg of *C*-fragment (▲) or 3 mg of *C*₂-fragment and 3 mg of *N*-fragment (■).

Fig. 2. Effect on the binding to CERBC receptors of different *C* fragments and ^{59}Fe *N* fragment

■, ^{59}Fe -*N* fragment in the presence of *C*-fragment; □, apo-(*C* fragment); ▲, *C_u* fragment; △, apo-(*C_u* fragment); ●, ^{59}Fe -*C* fragment alone.

Effect of various *C* half-molecules on the binding of iron-saturated *N* half-molecules to CERBC

^{59}Fe *N* Half-molecule alone binds very weakly, but in the presence of various *C* half-molecules it becomes tightly bound (Fig. 2), most likely to the whole protein (results not shown). The ability of the *N* and *C* fragments to dimerize together is not necessary; neither is it necessary for the *C* half-molecule to be iron-saturated.

Effect of various *N* half-molecules on the binding of iron-saturated *C* half-molecule to CERBC

^{59}Fe *C* half-molecule alone binds very weakly and, as with the ^{59}Fe -*N* half-molecule alone, it does not give the typical co-operative binding profile, i.e. a hyperbolic curve, but a straight line. In the presence of various unlabelled *N* half-molecules, the ^{59}Fe -*C* fragment (Fig. 3) behaves like the ^{59}Fe -*N* fragment as described above. In this case also dimerization of the *C* and *N* fragments is not required for binding to the CERBC to take place, neither is it necessary for the *N* half-molecule to be iron-saturated.

DISCUSSION

Recently, studies by Brown-Mason *et al.* (1987) have shown that *N* and *C* half-molecule fragments of ovotransferrin alone cannot bind to CERBC, in contrast with the previous report of Keung & Azari (1982). Furthermore, they claim that "the isolated half-molecules of ovotransferrin are able to reassociate in solution and that this reassociation has functional significance by allowing the complex to be recognized by the transferrin receptor".

We prepared non-dimerizing half-molecules, *N_u* and *C_u*, as well as the dimerizing forms, *N* and *C*, as described by Williams & Moreton (1988). The gel-filtration patterns clearly confirmed the different ability to associate in solution of both kinds of uncomplexable fragments. We cannot rule out that weak association forces between the two fragments are still present, but, nevertheless, they seem to be significantly weakened, as demonstrated by other experimental approaches (Williams & Moreton, 1988); in consequence this should be reflected in the features of ovotransferrin-fragment binding to the receptor. Testing these half-molecules for binding to CERBC receptors under different experimental conditions, we find that (i) the binding of the single fragments does not occur, but that (ii) reassociation in solution of fragments is not a prerequisite for binding to CERBC receptors. These conclusions were valid in all our experiments, even though the numbers of molecules bound per cell often changed slightly from one CERBC preparation to another, as already reported (Brown-Mason *et al.*, 1987). However, using the same cell stock, we have always observed a decrease in the maximum value of bound molecules per cell in the order: iron-saturated dimerizing fragments > apo-(dimerizing fragments) > iron-saturated uncomplexable fragments > apo-(uncomplexable fragments), regardless of the labelled fragmented tested.

Although an isolated iron-saturated *N* or *C* fragment will not bind tightly until a contralateral fragment is added, it does not matter whether the latter carried Fe(III) or not. It has already been reported that monoferric transferrin binds to the receptor less tightly than does diferric transferrin (Huebers *et al.*, 1983). Our data do not contradict this evidence, and it would be of interest

Fig. 3. Effect on the binding to CERBC receptors of different *N* fragments and ^{59}Fe -*C* fragment

■, ^{59}Fe *C* fragment in the presence of *N* fragment; □, apo-(*N* fragment); ▲, N_0 fragment; △, apo-(N_0 fragment); ●, ^{59}Fe -*N* fragment alone.

to compare the binding affinities of di- and mono-ferric ovotransferrin to CERBC. When the ^{125}I -labelled apo-fragments were tested in the presence of the contralateral monoferric fragment, we found binding of the fragment, but to a lower extent. This decrease is more evident for the apo-(*C*-fragment) than for the *N*-fragment; this confirms the finding of Mason & Mason (1987) that iodination of the *C* fragment causes a significant decrease in binding. A further aspect to be investigated concerns the efficiency of the mixtures in delivering iron into the cell, that is, iron uptake. The last basic conclusion that arises from our data and that of Brown-Mason & Woodworth (1984) is that the ovotransferrin receptor must have specific binding sites for the *N* and *C* domains of ovotransferrin.

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