Evolutionary significance of the renal excretion of transferrin half-molecule fragments

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It is generally thought that the duplicated structure of serum transferrin in vertebrates arose by gene duplication and fusion from a small ancestral protein. We have found that the isolated domains of transferrin are rapidly lost from the bloodstream via the kidneys. Therefore we suggest that the ancestral transferrin was not a serum protein or, alternatively, that it was not as small as the half-molecule.

Transferrin is the iron-transporting protein in the serum of vertebrates. Other closely related members of the transferrin class are ovotransferrin, which occurs in avian egg white, and lactoferrin, which occurs in external secretions of mammals. These proteins consist of a single polypeptide chain of mol.wt. about 80 000 (Greene & Feeney, 1968) that folds up into two compact structural regions or domains (Gorinsky *et al.*, 1979). Each domain carries a specific binding site for one iron atom. The domains are formed from the *N*- and *C*-terminal halves of the chain and they can be isolated after partial proteolysis as single-chain iron-binding fragments with mol.wts. about 40 000 (Williams, 1974, 1975). A notable feature of the primary structure of transferrin is the clear homology of the two halves, about 40% of the residues being identical (MacGillivray *et al.*, 1977; Jeltsch & Chambon, 1982; Williams *et al.* 1982). It was first suggested by Greene & Feeney (1968) that the two domains are descendants of a common ancestral protein and that gene duplication and fusion occurred early in the evolution of transferrin. The hypothetical ancestral protein is thought to have had a mol.wt. of about 40 000 and to have bound one atom of iron. Subsequently the two domains diverged in structure and iron-binding properties. The possibility that the ancestral transferrin was itself derived from the duplication of an even smaller protein has also been mentioned (MacGillivray *et al.*, 1977; Jeltsch & Chambon, 1982).

Considerable interest was aroused by the claim that at least one living species, the hagfish (*Eptatretus stoutii*), possesses a serum transferrin whose mol.wt. is much less than 80 000 (Palmour & Sutton, 1971). By using three different methods, the molecular weight of purified hagfish transferrin was estimated to be 44 000. Re-investigation (Aisen *et*

al., 1972), however, found the molecular weight of hagfish transferrin to be similar to that of other vertebrates (75 000–80 000). The absence of the small ancestral type of transferrin from living species is thought by some authors to pose a problem: Aisen & Listowsky (1980) write 'Why a two-sited protein should possess biological advantages enabling it to endure to the exclusion of its single-sited ancestor remains a mystery of transferrin biochemistry'.

Claude Bernard (1859) and several other nineteenth-century physiologists found that, after injection of egg white into the bloodstream of a mammal, rapid excretion of protein into the urine took place. Subsequent work showed that this phenomenon is closely connected with the size of the protein molecules (Bott & Richards, 1941). For many proteins of mol.wts. less than 70 000, the rate at which they filter through the glomerular membrane is inversely proportional to molecular diameter (Brenner & Rector, 1981). Several authors (Schultze & Heremans, 1966; Putnam, 1975) have proposed that this dependence explains the fact that most plasma proteins have mol.wts. above 60 000, although the tubular re-absorption of filtered protein is important in some cases. If this theory is valid for transferrin, it would be expected that a molecule resembling the proposed ancestor would be rapidly removed from the blood via the kidney, whereas no loss of the duplicated transferrin should occur. The present paper shows that this expectation is readily confirmed.

Experimental and results

The iron complexes of the *N*- and *C*-terminal half-molecule fragments of hen ovotransferrin were prepared as described previously (Williams, 1974, 1975). They gave single bands on sodium dodecyl

sulphate/polyacrylamide-gel electrophoresis in the presence of mercaptoethanol, and the estimated mol.wts. were 41000 (*N*-terminal) and 43700 (*C*-terminal). The molecular weight of the ovotransferrin was 79000. Three groups of ten adult female mice (strain CBA/H/Ig^b) were taken. In the first group each animal received an intravenous injection of 5 mg of ovotransferrin in 0.5 ml of saline (0.9% NaCl); in the second group, 5 mg of the *C*-terminal half-molecule was injected; in the third group, 5 mg of the *N*-terminal half-molecule. The urine from the mice in each group was collected after 1 h and pooled. The urine from the mice that had received ovotransferrin showed the normal yellow colour, whereas that from the mice that had received the half-molecules was a distinct orange colour (the iron complexes are pink in colour). The presence of the transferrin half-molecules in the urine was confirmed by the Ouchterlony method (Fig. 1) with a rabbit antiserum against hen ovotransferrin. The urine from the mice injected with whole ovotransferrin gave no reaction. Sodium dodecyl

sulphate/polyacrylamide-gel electrophoresis showed that the half-molecules excreted in the urine had the same molecular weights as the material injected. Quantitative single radial-immunodiffusion assay (Mancini *et al.*, 1965) showed that, in the case of both the half-molecules, 30% of the material injected had been excreted during the first hour.

Discussion

In the retention of the duplicated protein within the circulation and the rapid excretion of the half-molecules, transferrin conforms to the general relation between molecular size and glomerular filtration for proteins. Although our observations are not conclusive, it seems unlikely that any vertebrate species will be found to possess a serum transferrin with a mol.wt. as low as 40000. The half-molecule fragments of ovotransferrin used in these experiments are similar in size to the corresponding domains of human serum transferrin (Evans & Williams, 1978). Transferrin is only known to occur in vertebrates, and it was presumably acquired by an ancestor resembling the protochordates, although living representatives of this group apparently have no transferrin-like blood proteins (Hawkins *et al.*, 1980). If at its first appearance transferrin was indeed as small as has been suggested, we do not know how it can have been retained in the bloodstream.

The same problem applies to other plasma proteins that are thought to have undergone duplication during their evolution. A possible solution in the case of immunoglobulin is provided by the discovery of a low-molecular-weight membrane-bound glycoprotein which appears to be homologous to a single domain of immunoglobulin (Cohen *et al.*, 1981). By analogy we suggest that the ancestral transferrin may have been a membrane-bound metal-receptor protein.

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Fig. 1. Ouchterlony test for the presence of transferrin half-molecules

The centre well contained rabbit antiserum against ovotransferrin. Wells 1-4 contained urine samples from mice that received intravenous injections of the *C*-terminal fragment of transferrin (wells 1 and 4), the *N*-terminal fragment of transferrin (well 2) or intact ovotransferrin (well 3). Wells 5 and 6 contained standard preparations of the *C*-terminal and *N*-terminal fragments respectively. Although the fragments react with anti-ovotransferrin, they do not share antigenic determinants (Williams, 1974, 1975).

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