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The electrophoresis of transferrins in urea/polyacrylamide gels

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The denaturation of transferrin by urea has been studied by (*a*) electrophoresis in polyacrylamide gels incorporating a urea gradient, (*b*) measurements of the loss in iron-binding capacity and (*c*) u.v. difference spectrometry. In human serum transferrin and hen ovotransferrin the *N*-terminal and *C*-terminal domains of the iron-free protein were found to denature at different urea concentrations.

Makey & Seal (1976) showed that partially iron-saturated human transferrin can be resolved into four bands by polyacrylamide-gel electrophoresis in a buffer containing 6 M-urea. The slowest and fastest components represent the apoprotein and the diferric form of the protein respectively, and the two intermediate components represent the two monoferric forms of the protein. The identification of the two intermediate components was achieved by Evans & Williams (1978). Partial saturation of human serum transferrin with iron(III) nitrilotriacetate was found to give rise to the slower intermediate component, and proteolytic digestion of this substance produced an iron-containing fragment of mol.wt. 43000 that contained carbohydrate. On the other hand, partial saturation with other iron donors, including $FeCl_3$, gave rise to the faster intermediate component, and on proteolytic digestion this substance yielded an iron-containing fragment of mol.wt. 36000 that was devoid of carbohydrate. Since the amino-acid-sequence determinations of MacGillivray *et al.* (1977) had shown that carbohydrate is present only in the *C*-terminal half of the polypeptide chain, Evans & Williams (1978) inferred that the slower intermediate component represents the monoferric transferrin with iron in the *C*-terminal binding site (TfFe) and that the faster intermediate band represents the *N*-terminal monoferric transferrin (FeTf). Partially iron-saturated hen ovotransferrin also gives four bands upon polyacrylamide-gel electrophoresis in urea-containing buffers, and from similar evidence these can be identified as corresponding to the iron-free protein, the *C*-terminal monoferric transferrin (OTFe), the *N*-terminal monoferric transferrin (FeOT) and the diferric transferrin in the order of increasing electrophoretic mobility (Williams *et al.*, 1978).

This method of electrophoresis has proved useful in determining the distribution of iron between the two binding sites of human transferrin (Evans & Williams, 1978; Aisen *et al.*, 1978; Leibman & Aisen, 1979; Williams & Moreton, 1980) and of hen ovotransferrin (Williams *et al.*, 1978), since Aisen *et al.* (1978) have shown that no rearrangement of bound iron occurs during electrophoresis.

Electrophoretic separations of transferrins bearing different numbers of iron atoms in non-denaturing buffer solutions can be obtained as a result of the increased numbers of negative charges on molecules carrying iron (Aisen *et al.*, 1966). The separation of the different forms of transferrin in urea-containing buffers is likely to depend on two further effects. The first is the increased stability of iron–protein complexes to urea denaturation (Azari & Feeney, 1958). Secondly, as Makey & Seal (1976) suggested, the magnitudes of the decreases in electrophoretic mobility caused by the unfolding of the two domains of the protein may not be equal. Although there have been many quantitative studies of the denaturation of transferrin by urea (e.g. Glazer & McKenzie, 1963; Yeh *et al.*, 1979), there appears to be no information about possible differences between the *N*-terminal and *C*-terminal domains.

Creighton (1979) showed that the denaturation of proteins by urea can be studied by electrophoresis of a band of protein through a slab of gel in which there is a gradient of urea concentration perpendicular to the direction of electrophoresis. With several proteins, curves were obtained that were consistent with a rapidly reversible denaturation process in which only the native protein and the fully unfolded protein were detectable.

In the present paper we describe the application of

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urea-gradient/polyacrylamide-gel electrophoresis to several transferrins and show that the unfolding of the two domains occurs in two distinct steps.

Materials and methods

Transferrins

Human serum transferrin was isolated from Cohn IV fraction as described previously (Evans & Williams, 1978). Hen and duck ovotransferrins were isolated from the egg whites as described by Williams (1968). Pig transferrin and horse transferrin were isolated by the method of Graham & Williams (1975). Rat and rabbit transferrin samples were gifts from Mr. S. Heaphy of this department. Bovine transferrin was a gift from Dr. J. Brock, Department of Bacteriology, University of Glasgow, Glasgow, Scotland, U.K. The removal of iron from transferrin was carried out as described by Evans & Williams (1978).

Samples of human serum and orang-utan (*Pongo pygmaeus*) serum (the latter kindly supplied by Dr. C. Pearson, Department of Veterinary Medicine, University of Bristol, Langford, Bristol, U.K.) were also examined by the urea/polyacrylamide-gel electrophoresis after treatment with rivanol to remove the bulk of the serum proteins (Evans & Williams, 1978).

Polyacrylamide-gel electrophoresis

Electrophoresis in 6M-urea was carried out as described by Williams *et al.* (1978). Urea-gradient/polyacrylamide gels were prepared essentially as described by Creighton (1979). In both cases gels were 1.5mm thick. A linear gradient from 0 to 6M-urea was produced by using a multichannel peristaltic pump. Creighton (1979) used a shallow gradient of acrylamide concentration in the opposite direction to the urea gradient in order to compensate for a retardation in electrophoretic mobility due to the urea but not associated with unfolding of the protein. This step was not used in our experiments where the acrylamide and *NN'*-methylenebisacrylamide concentrations were kept constant at 6.5% (w/v) and 0.172% (w/v) respectively.

Protein samples were dissolved in reservoir buffer containing 0.01% Bromophenol Blue and 10% (w/v) glycerol to give a protein concentration of 1mg/ml. The sample was applied to the top of the gel at 10μl/cm of gel. When serum samples were used, 0.1ml of serum was mixed with 0.8ml of reservoir buffer containing 0.375% (w/v) rivanol and 10% (w/v) glycerol. After centrifugation the supernatant solution was applied to the gel at 50μl/cm of gel. Electrophoresis was carried out for 17h at 100V, and gels were stained with Coomassie Blue R250.

Addition of iron to transferrin in the presence of urea

The denaturation of iron-free transferrin by urea was monitored by testing the ability of the protein to form a complex with iron(III) nitrilotriacetate in the presence of different concentrations of urea. The formation of complexes was detected by the A_{470} and by electrophoresis in polyacrylamide gels containing 6M-urea.

Iron-free hen ovotransferrin or human transferrin were dissolved in 0.05M-$NaHCO_3$ containing different concentrations of freshly deionized urea. The protein concentration was between 3 and 10mg/ml in different experiments. A calculated amount of iron(III) nitrilotriacetate to give 100% saturation was added. Samples were left overnight at 20°C before measuring A_{470} and performing polyacrylamide-gel electrophoresis.

U.v. spectra

The denaturation of iron-free transferrin by urea was also monitored by u.v. difference spectroscopy. The protein was dissolved in the electrophoresis reservoir buffer (Tris/EDTA/boric acid, pH8.4) at a concentration of 0.4mg/ml, and a stock solution of deionized 8M-urea was added to give the desired urea concentration. The difference spectrum obtained between the protein in the urea-containing buffer and the protein in the absence of urea was similar to that found by Glazer & McKenzie (1963). Spectra were recorded on a Pye–Unicam SP. 8-100 instrument. The height of the difference peak at 292nm was used as a measure of protein unfolding.

Results

Electrophoresis of human transferrin in urea-gradient/polyacrylamide gels

Fig. 1 shows that increasing urea concentration from 0 to 6M decreases the electrophoretic mobility of iron-free transferrin to about 43% of its original value in two distinct steps. The first step occurred over a wider range of urea concentrations (approx. 2.8–4.0M) than the second (approx. 4.1–4.5M), but approximately equal amounts of retardation occurred in each step. Thus at 4.0M-urea the mobility is 70% of the value at 2.8M-urea and at 4.5M-urea it is 65% of the value at 4.0M-urea.

Fig. 2 shows the pattern given by a partially iron-saturated sample of human transferrin in which all four forms of the protein were present. The diferric form showed no denaturation steps over the range of urea concentration used. The monoferric complex, in which the single iron atom occupies the *N*-terminal binding site (FeTf), showed a single denaturation step that coincided with the first of the

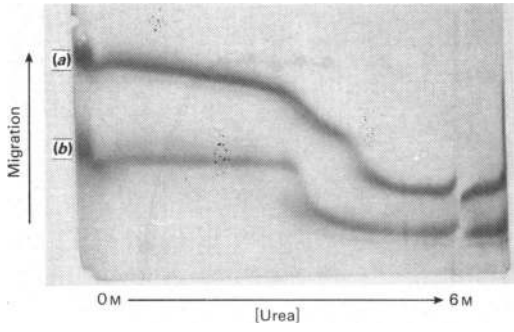

Fig. 1. *Electrophoresis of iron-free human transferrin and hen ovotransferrin in a urea-gradient/polyacrylamide gel*
A mixture of the two proteins was electrophoresed in the same gel. (*a*) Human transferrin; (*b*) hen ovotransferrin.

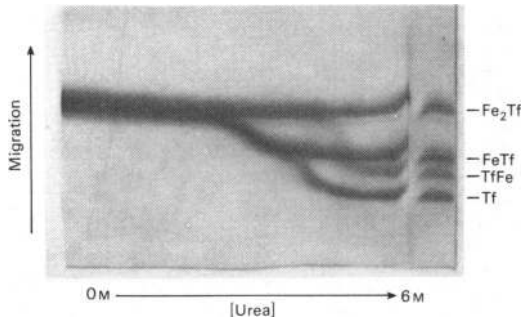

Fig. 2. *Electrophoresis of partially iron-saturated human transferrin on a urea-gradient/polyacrylamide gel*
A partially iron-saturated sample of human transferrin containing all four forms of the protein was applied to the gel. It was prepared by adding small amounts of iron, as $Fe(II)SO_4$ and iron(III) nitrilotriacetate, to apotransferrin (Evans & Williams, 1978). The diferric protein is labelled 'Fe_2Tf', the *N*-terminal monoferric complex 'FeTf', the *C*-terminal monoferric complex 'TfFe' and the apoprotein 'Tf'.

two denaturation steps for iron-free transferrin (approx. urea concentration at the midpoint of the step, 3.4 M). This suggests that this step represents the denaturation of the iron-free *C*-terminal domain of the protein. The monoferric complex with an iron atom bound to the *C*-terminal site (TfFe) also showed a single denaturation step at a slightly higher urea concentration than the second of the two denaturation steps for the iron-free protein (approx. urea concentration at the mid-point of the step, 4.8 M, as compared with 4.3 M). The unfolding of TfFe complex decreased its electrophoretic mobility

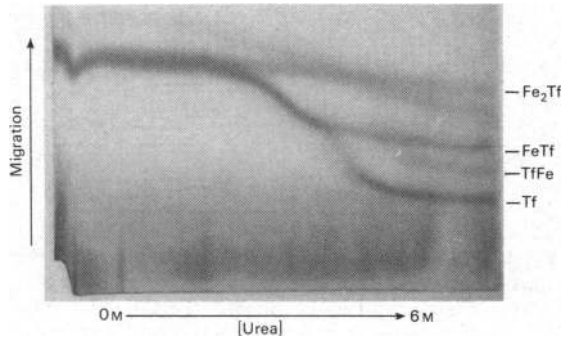

Fig. 3. *Electrophoresis of a fresh sample of human serum after treatment with rivanol on a urea-gradient/polyacrylamide gel*
The transferrin bands are labelled as in Fig. 2.

to about 59% of its value at urea concentrations below 4.2 M, causing the TfFe complex to migrate more slowly than the FeTf complex. Again it can be seen that the unfolding of the *N*-terminal domain occurs over a narrower range of urea concentration than the unfolding of the *C*-terminal domain.

When fresh human serum was treated with rivanol and subjected to urea-gradient electrophoresis, the pattern obtained was essentially the same as that given by purified transferrin (Fig. 3). The concentration of the *N*-terminal monoferric form is greater than that of the *C*-terminal monoferric form, as Leibman & Aisen (1979) and Williams & Moreton (1980) have shown recently.

Electrophoresis of hen ovotransferrin in urea-gradient/polyacrylamide gels

Fig. 1 shows that, in a urea-gradient/polyacrylamide gel, the electrophoretic mobility of iron-free ovotransferrin is decreased to about 39% of its value at low urea concentrations. This decrease appears to take place as a single step that occurs at the same urea concentration as the first step of iron-free human transferrin (approx. 3.4 M), although the slope of the line is steeper in the case of the hen protein.

A partially iron-saturated sample in which all four protein components were present gave the pattern shown in Fig. 4. The diferric protein showed no marked change in electrophoretic behaviour. The *C*-terminal monoferric complex (OTFe) showed a single-step decrease in mobility to 50% of its original value. This step occurred at the same urea concentration as did the retardation of the iron-free protein. The *N*-terminal monoferric complex (FeOT) showed a much smaller decrease in mobility, to 68% of its original value, and this took place at a higher urea concentration (approx. 3.8 M) than did the unfolding of the iron-free protein.

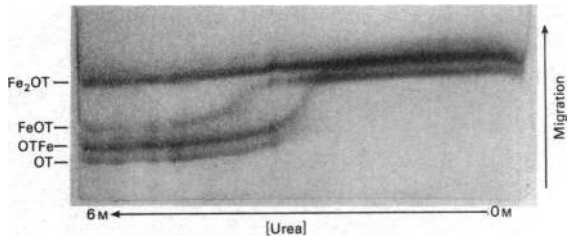

Fig. 4. *Electrophoresis of partially iron-saturated hen ovotransferrin on a urea-gradient/polyacrylamide gel*
The diferric protein is labelled 'Fe_2OT', the *N*-terminal monoferric complex 'FeOT', the *C*-terminal monoferric complex 'OTFe' and the apoprotein 'OT'. This mixture was prepared by adding a small amount of iron(III) nitrilotriacetate to ovotransferrin as prepared by the method of Williams (1968).

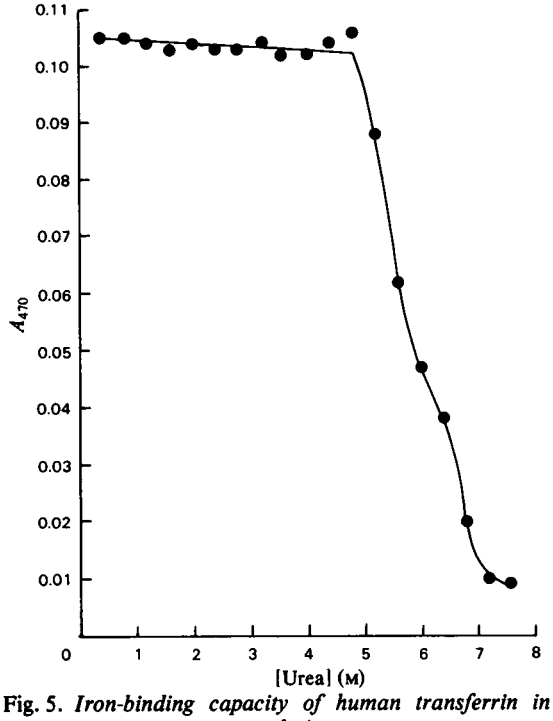

Fig. 5. *Iron-binding capacity of human transferrin in urea solutions*
Saturating amounts of iron were added to solutions of the iron-free protein (2.9 mg/ml) containing different concentrations of urea. For conditions, see the text.

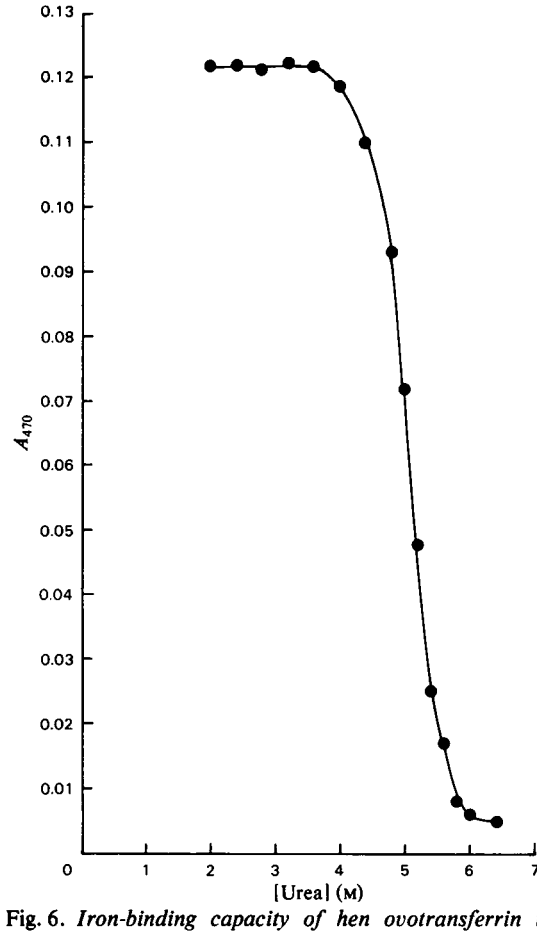

Fig. 6. *Iron-binding capacity of hen ovotransferrin in urea solutions*
Saturating amounts of iron were added to solutions of the iron-free protein (2.9 mg/ml) containing different concentrations of urea. For conditions, see the text.

Electrophoresis of other transferrins

On electrophoresis in urea-gradient/polyacrylamide gels the iron-free transferrins of rat, rabbit, pig, horse and cow, and duck ovotransferrin, all showed an apparently single-step decrease in mobility to 32–49% of the original value. Rivanol-treated samples of serum from the orang-utan, on the other hand, gave a pattern similar to that of human serum, in which the decrease in mobility of iron-free transferrin showed two distinct steps.

Ability of transferrin to bind iron in the presence of urea

When a saturating dose of iron(III) nitrilotriacetate was added to iron-free human transferrin in the presence of different concentrations of urea, the ability of the protein to form a coloured complex was destroyed in the concentration range 5.0–7.2 M (Fig. 5).

Polyacrylamide-gel electrophoresis in 6 M-urea showed that from 5.2 to 6.8 M-urea the *N*-terminal monoferric complex (FeTf) was present as an intermediate component. There was no sign of the

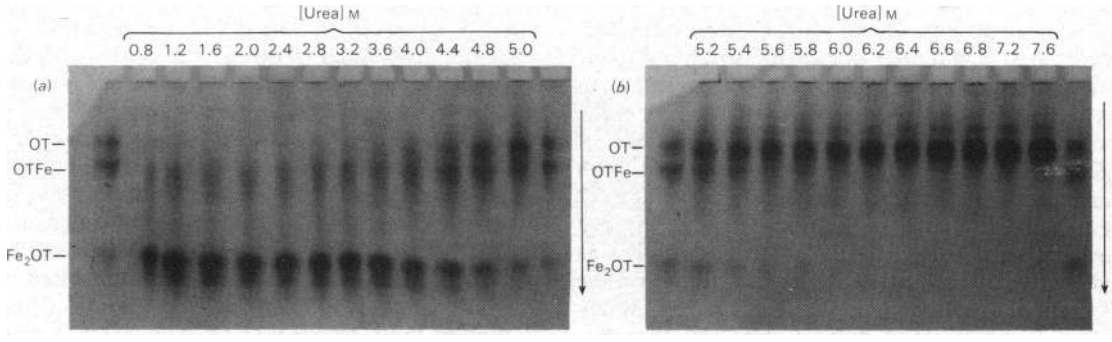

Fig. 7. *Electrophoresis of ovotransferrin after addition of iron to the protein in different urea concentrations*
A saturating dose of iron was added to ovotransferrin dissolved in urea solution. Electrophoresis in a polyacrylamide gel containing 6 M-urea was carried out 3 h later. 'M' denotes a marker sample containing apoprotein (OT), the *C*-terminal monoferric complex (OTFe) and the diferric protein (Fe_2Tf). (*a*) Urea concentrations 0.8–5.0 M; (*b*) urea concentrations 5.2–7.6 M.

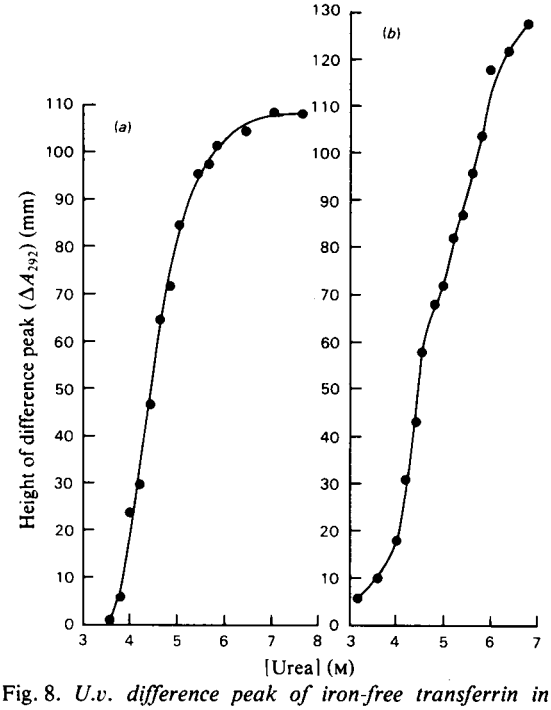

Fig. 8. *U.v. difference peak of iron-free transferrin in urea solutions*
Difference spectra of transferrin in urea solutions against transferrin in urea-free solutions were measured. The protein concentration was 0.4 mg/ml. (*a*) Hen ovotransferrin; (*b*) human transferrin.

C-terminal monoferric complex (TfFe). This showed that the iron-binding capacity of the *C*-terminal domain of human transferrin is lost before that of the *N*-terminal domain. Hen ovotransferrin lost its ability to bind iron in the range 4.0–6.0 M-urea (Fig. 6), and electrophoresis showed that, over this range, the *C*-terminal monoferric complex (OTFe) was present as the sole intermediate (Fig. 7). Therefore in this protein, despite an apparently single-step denaturation process (as judged by urea-gradient/polyacrylamide-gel electrophoresis and by the loss of iron-binding capacity), the *N*-terminal domain is denatured before the *C*-terminal domain.

U.v. difference spectroscopy

Fig. 8 shows that, for both iron-free human transferrin and iron-free hen ovotransferrin, increase in the height of the 292 nm difference peak occurs over a wide range of urea concentrations: aprox. 3.0–7.0 M for human transferrin and 3.5–6.5 M for ovotransferrin.

Discussion

In these experiments the urea denaturation of iron-free transferrin has been studied by u.v. measurements, gel electrophoresis and iron-binding measurements. The main finding is that these three methods reveal different aspects of the denaturation of transferrin. U.v. measurements show that unfolding of the protein occurs over a wide range of urea concentrations (3–7 M-urea for human transferrin and 3.5–6.5 M-urea for ovotransferrin). The decrease in electrophoretic mobility takes place in the lower part of this range of urea concentrations. In human transferrin, two distinct steps occurred at 2.8–4.0 M-urea and 4.1–4.5 M-urea. From the behaviour of the monoferric complexes (see below) it is suggested that the first step is due to the unfolding of the *C*-terminal domain and the second step to that of the *N*-terminal domain. In ovotransferrin a single-step decrease in mobility was seen, with its mid-point at 3.4 M-urea, and this probably represents the simultaneous unfolding of both domains. The loss of ability to bind iron, as judged by A_{470}, takes place in the upper part of the urea

concentration range (5–7.2 M-urea for human transferrin and 4–6 M-urea for ovotransferrin). Thus iron-binding can still occur in a concentration of urea causing maximum decrease in electrophoretic mobility. Moreover, there is sequential loss of iron-binding ability by the two domains in both human transferrin and ovotransferrin (Fig. 7), despite the fact that, in the latter protein, the two domains appear to denature simultaneously, as judged by electrophoresis. In human transferrin the *C*-terminal domain loses its iron-binding ability before the *N*-terminal domain does. In ovotransferrin the order is reversed.

In the denaturation of the monoferric transferrin complexes the unfolding of the iron-free domain is assumed to be responsible for the decrease in electrophoretic mobility, since the diferric protein complexes appear completely to resist unfolding as judged by this method. Fig. 2 shows that FeTf denatures at the same urea concentration as the first step for iron-free Tf. It is likely, therefore, that in both cases this step is due to the unfolding of the *C*-terminal domain. There is also evidence (Figs. 2 and 4) that, for some monoferric complexes, the iron-free domain received some protection against urea denaturation from the presence of an iron atom in the other domain. Thus, for human transferrin, the *N*-terminal domain in the monoferric complex TfFe unfolds at a higher urea concentration than does the *N*-terminal domain in the iron-free protein. In ovotransferrin the *C*-terminal domain is more stable in the monoferric complex FeOT than in the iron-free protein. On the other hand, there appears to be no protection of the *C*-terminal domain in FeTf or of the *N*-terminal domain in OTFe. The chemical basis for this asymmetrical effect is completely unknown. The resolution of the two monoferric complexes during electrophoresis in 6 M-urea/polyacrylamide gels depends on the fact that, in both human transferrin and hen ovotransferrin, the unfolding of the *N*-terminal domain causes a greater decrease in mobility than does the unfolding of the *C*-terminal domain.

Structural differences that might explain the different stabilities of the two domains towards the effects of urea are unknown. Disulphide bridges are thought to be important in maintaining stability, but in ovotransferrin the half-cystine contents of the *N*- and *C*-terminal domains are approximately equal (Williams, 1975), whereas, in human transferrin, MacGillivray *et al.* (1977) found sixteen half-cystine residues in the *C*-terminal half of the polypeptide chain and only eight in the *N*-terminal half. However, Evans & Williams (1978) found nearly equal half-cystine contents for the *N*-terminal and *C*-terminal fragments of human transferrin.

Differential calorimetry has been used to obtain information on the thermal denaturation of transferrin. Donovan & Ross (1976) found that iron-free human transferrin showed two peaks of thermal denaturation, at 62 and 72°C, and suggested that these represented the denaturation of the two domains of the protein. It is not known which domain corresponds to each peak of thermal denaturation. On the other hand, iron-free hen ovotransferrin gave a single peak of denaturation at 63°C (Donovan & Ross, 1975). These results are similar to those on urea denaturation reported here. Evans *et al.* (1977) studied the thermal denaturation of the *N*-terminal and *C*-terminal iron-binding fragments obtained from ovotransferrin by partial proteolysis. They found that the *N*-terminal fragment denatured at 56.5°C and the *C*-terminal fragment at 60°C. Although data on fragments cannot be compared directly with data on the whole protein, the calorimetry is consistent with the finding that the *N*-terminal domain of the ovotransferrin denatures before the *C*-terminal in urea solutions.

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References

Aisen, P., Leibman, A. & Reich, H. A. (1966) *J. Biol. Chem.* **241**, 1666–1671

Aisen, P., Leibman, A. & Zweier, J. (1978) *J. Biol. Chem.* **253**, 1930–1937

Azari, P. R. & Feeney, R. E. (1958) *J. Biol. Chem.* **232**, 293–302

Creighton, T. E. (1979) *J. Mol. Biol.* **129**, 235–264

Donovan, J. W. & Ross, K. D. (1975) *J. Biol. Chem.* **250**, 6026–6031

Donovan, J. W. & Ross, K. D. (1976) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35**, 1608

Evans, R. W. & Williams, J. (1978) *Biochem. J.* **173**, 543–552

Evans, R. W., Donovan, J. W. & Williams, J. (1977) *FEBS Lett.* **83**, 19–22

Glazer, A. N. & McKenzie, H. A. (1963) *Biochim. Biophys. Acta* **71**, 109–123

Graham, I. & Williams, J. (1975) *Biochem. J.* **145**, 263–279

Leibman, A. & Aisen, P. (1979) *Blood* **53**, 1058–1065

MacGillivray, R. T. A., Mendez, E. & Brew, K. (1977) in *Proteins of Iron Metabolism* (Brown, E. B., Aisen, P., Fielding, J. & Crichton, R. R., eds.), pp. 133–141, Greene and Stratton, New York

Makey, D. G. & Seal, V. S. (1976) *Biochim. Biophys. Acta* **453**, 250–256

Williams, J. (1968) *Biochem. J.* **108**, 57–67

Williams, J. (1975) *Biochem. J.* **149**, 237–244

Williams, J. & Moreton, K. (1980) *Biochem. J.* **185**, 483–488

Williams, J., Evans, R. W. & Moreton, K. (1978) *Biochem. J.* **173**, 535–542

Yeh, Y., Iwai, S. & Feeney, R. E. (1979) *Biochemistry* **18**, 882–889