Structural Evidence for a pH-Sensitive Dilysine Trigger in the Hen Ovotransferrin N-Lobe: Implications for Transferrin Iron Release‡

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ABSTRACT: Members of the transferrin family of proteins are involved in Fe^{3+} transport (serum transferrins) and are also believed to possess antimicrobial activity (ovotransferrins and lactoferrins). The structure of the monoferric N-terminal half-molecule of hen ovotransferrin, reported here at 2.3-Å resolution, reveals an unusual interdomain interaction formed between the side-chain NZ atoms of Lys 209 and Lys 301, which are 2.3 Å apart. This strong interaction appears to be an example of a low-barrier hydrogen bond between the two lysine NZ atoms, both of which are also involved in a hydrogen-bonding interaction with the aromatic ring of a tyrosine residue. Crystals of the protein were grown at pH 5.9, which is well below the usual $pK_a \sim 10$ for a lysine side chain. We suggest that the pK_a of either one or both of these residues lies below the pH of the structure determination and is, therefore, not positively charged. This finding may serve to explain, on a molecular basis, the pH dependence of transferrin Fe^{3+} release. We propose that uptake of the Fe^{3+}–transferrin complex into an acidic endosome (*viz.*, pH ~5.0) *via* receptor-mediated endocytosis will result in the protonation of both lysine residues. The close proximity of the two resulting positive charges, and their location on opposite domains of the N-lobe, might well be the driving force that opens the two domains of the protein, exposing the Fe^{3+} ion and facilitating its release. Examination of amino acid sequences of other transferrins indicates that similar pH-sensitive dilysine triggers are possible in the N-lobe, but not in the C-lobe, of most serum transferrins although such interactions have not been reported. Dilysine triggers are not possible in the C-lobe of hen ovotransferrin or in either lobe of most lactoferrins.

The transferrins (Aisen & Listowsky, 1980; Huebers & Finch, 1987; Aisen, 1989; Harris & Aisen, 1989; Baker & Lindley, 1992) are glycoproteins composed of a single polypeptide chain ($M_w \sim 80\,000$). They are bilobal with two structurally similar lobes, presumed to have arisen by gene duplication and fusion, sharing sequence identity of ~40%. The short section of chain connecting the lobes can be cleaved proteolytically to yield active N- and C-terminal half-molecules ($M_w \sim 40\,000$) (Oe et al., 1988). Each lobe (or half-molecule) is further divided into two similarly sized domains that are connected by two antiparallel β-strands which act as a hinge between them. One Fe^{3+} ion is bound in the interdomain cleft (Figure 1A). Crystal structures of diferric human lactoferrin (Anderson et al., 1989), monoferric human serum transferrin (Zuccola, 1993), diferric rabbit serum transferrin (Bailey et al., 1988), and its monoferric N-terminal half-molecule (Sarra et al., 1990) have been determined. In each of these structures the two domains are closed over their Fe^{3+} ions except for the N-lobe of human transferrin, which is in the open apo form. The human apolactoferrin structure has also been determined (Anderson et al., 1990) and possesses an open N-lobe and closed C-lobe. Neither lobe bears an Fe^{3+} ion. The structure of the Fe^{3+}-containing quarter-molecule of domain 2 from the duck ovotransferrin N-lobe has also appeared (Lindley et al., 1993).

The serum transferrins bind Fe^{3+} extremely tightly at pH values above ~6 but release it relatively readily below this value. At pH 7.4 the effective affinity constant is $\sim 10^{22}\ M^{-1}$ (Aisen & Listowsky, 1980). Receptor-mediated endocytosis of the Fe^{3+}–transferrin complex into an acidic endosome (pH ~5; Yamashiro et al., 1983), with subsequent release of Fe^{3+}, is the generally accepted mechanism for Fe^{3+} delivery to a cell (van Renswoude et al., 1982; Rao et al., 1983; Huebers & Finch, 1987; Aisen, 1989). *In vitro*, the two lobes of the serum transferrins display somewhat different Fe^{3+} release properties. The N-lobe of human serum transferrin, for example, will release Fe^{3+} at pH ~5.7 while the C-lobe retains Fe^{3+} to pH ~4.8 (Princiotto & Zapolski, 1975; Lestas, 1976; Baldwin et al., 1982). The lactoferrins, however, reportedly retain Fe^{3+} to much lower pH values (Montreuil et al., 1960). These observations are in accord with the general belief that the role of the serum transferrins is that of Fe^{3+} transport while the role of the lactoferrins is believed to be as antimicrobials in which capacity they act simply as Fe^{3+} sequesterers. The serum transferrins, therefore, require a mechanism for Fe^{3+} release whereas the lactoferrins have no such requirement. Here we report the crystal structure of the monoferric N-terminal half-molecule of hen ovotransferrin at 2.3-Å resolution and suggest that a close 2.3-Å interaction between the side-chain NZ atoms of two lysine residues may provide the molecular explanation for the relative ease with which the N-lobe of the serum transferrins releases Fe^{3+} at pH values below ~6.

EXPERIMENTAL PROCEDURES

Crystallization. The N-terminal half-molecule of hen ovotransferrin, consisting of residues 1–332 with no attached carbohydrate, was isolated and purified as described by Oe et al. (1988). Orange crystals were grown by a modification of the method described by Mikami and Hirose (1990). The

‡ Coordinates and observed structure factors for the crystal structure of the monoferric N-terminal half-molecule of hen ovotransferrin have been deposited with the Brookhaven Protein Data Bank as entry 1NNT.

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hanging-drop vapor-diffusion method was employed where 5 μL of protein solution (59 mg/mL, pH 5.9, 0.02 M acetate buffer, 0.01% NaN_3) was mixed with 5 μL of precipitant solution [4–5% poly(ethylene glycol) 6000, pH 5.9, 0.02 M acetate buffer] on a silanized coverslip that was inverted and sealed above 1 mL of the precipitant solution. The drops were seeded if crystals had not appeared in 3–4 days. Crystals then grew within 2–3 weeks but began to crack up after about 4 weeks, an effect that might be due to a slight lowering of pH with time. Room temperature was employed throughout. Crystals are orthorhombic, space group $P2_12_12_1$ with a = 46.94 Å, b = 91.79 Å, c = 75.82 Å, and Z = 4.

Data Collection and Processing. X-ray data were collected using Cu Kα radiation (λ = 1.5418 Å) with a Siemens area detector coupled to a Rigaku RU-200 rotating-anode generator. A total of 59 108 reflections were collected to 2.3-Å resolution from one crystal. Data reduction was carried out with the XENGEN package of programs (Siemens Analytical X-ray Instruments, Inc., Madison, WI). Equivalent reflections were averaged (R_{sym} = 0.090 on intensity), yielding 14 266 unique reflections (94% complete). The mean $I/\sigma(I)$ for the data set was 8.6.

Structure Solution. The structure was solved by molecular replacement. The search model consisted of residues 1–340 of diferric rabbit serum transferrin (Bailey et al., 1988) with the Fe^{3+} ion excluded. The program MERLOT (Fitzgerald, 1988) was used to solve the rotation function and yielded a unique solution that was 5.3σ above the mean employing the data between 15- and 4.5-Å resolution. The X-PLOR program package (Brünger, 1992) was employed to solve the translation function using the data in the 15–4-Å resolution range. PC refinement (Brünger, 1992) of the appropriately rotated search model, as a rigid group, was carried out prior to the translation search. The correct solution was the top peak from this search at 10.4σ above the mean with the next peak at 8.6σ. The molecular replacement solution was confirmed with a native anomalous-difference Patterson map that gave the correct location for Fe^{3+}.

Structure Refinement. Rigid body refinement of the search model, rotated and translated according to the molecular replacement solutions, yielded an R-factor of 0.476 for the data between 12- and 3-Å resolution. Data with $I > 2\sigma(I)$ were used in all refinements. The model was then assigned the correct sequence by replacing residues with the model-building program TOM, derived from FRODO (Jones, 1985). Refinement by simulated annealing with molecular dynamics using a slow-cooling protocol similar to that of Brünger et al. (1990), from 3000 to 300 K, yielded R = 0.266 for the data between 9- and 3-Å resolution. Several rounds of simulated annealing with X-PLOR (Brünger, 1992), followed by manual model building, were carried out to improve the model. Once it became available, the human lactoferrin structure (Anderson et al., 1989), which is more closely related to the ovotransferrin structure than is the rabbit serum structure, aided in clarifying certain loops of the ovotransferrin structure. Final refinement with TNT (Tronrud et al., 1988) converged at an R-factor of 0.160 for the 8965 reflections with $I > 2\sigma(I)$ in the 20–2.3-Å resolution range [R = 0.176 for the 10 520 reflections with $I > \sigma(I)$]. The final model consists of the 2533 non-hydrogen atoms of the protein, 108 water molecules, 1 Fe^{3+} ion, and 1 CO_3^{2-} anion. Root-mean-square deviations of bond lengths and angles from ideality are 0.015 Å and 2.25°, respectively. Residues 1–4 are disordered and are not included in the model. All remaining residues lie within "allowed" regions of the Ramachandran plot, the only exception being Leu 299 which lies in a similar "disallowed" region in lactoferrin (Anderson et al., 1989).

RESULTS AND DISCUSSION

The polypeptide fold of the hen ovotransferrin N-lobe is similar to that observed in the lobes of other transferrins with bound Fe^{3+} (Bailey et al., 1988; Anderson et al., 1989; Sarra et al., 1990; Zuccola, 1993). Both domains of the single lobe consist of a central irregular twisted β-sheet covered on either side by connecting loops and helices (Figure 1A). The metal binding sites of the transferrins are unusual in that four of the Fe^{3+} ligands are provided by side chains from residues that are remote from one another in the protein sequence. Ovotransferrin possesses a metal binding site (Figure 1B) similar to those previously reported with Asp 60 and Tyr 191 being provided by domains 1 and 2, respectively, while Tyr 92 and His 250 are provided by one each of the β-strands of the hinge. The remaining two Fe^{3+} coordination sites are occupied by bidentate CO_3^{2-} (or HCO_3^-), the synergistic anion, so-called because of the requirement of both Fe^{3+} and anion for binding to the protein (Huebers & Finch, 1987; Harris & Aisen, 1989).

The refined high-resolution ovotransferrin structure reveals a novel interaction between nitrogen atoms of Lys 209 (domain 2) and Lys 301 (domain 1) (Figures 1 and 2). The NZ atoms of these two lysines are 2.3 Å apart, making it unlikely that both residues are positively charged in the present closed structure of ovotransferrin. Presumably, either one or both of these lysine residues have a pK_a value below the pH 5.9 of the current structure determination and are neutral. Except for one of the tyrosinate ligands to Fe^{3+}, namely, Tyr 191, there are no negatively charged residues in the immediate vicinity of the two lysines that could neutralize two positive charges (Figure 1B,C). Release of Fe^{3+} from the transferrins is believed to occur within an acidic endosome (van Renswoude et al., 1982; Rao et al., 1983) which, at pH ~5.0, should result in the protonation of both lysine residues to produce two positive charges. Any mechanism for Fe^{3+} release from the transferrins, at any pH, presumably requires the initial opening of the two domains to expose the Fe^{3+} ion (Baldwin et al., 1982; Cowart et al., 1982; Harris, 1984; Kretchmar & Raymond, 1988). The side chains of Lys 209 and Lys 301 contact one another across the interdomain cleft, being located between the Fe^{3+} ion and the two β-strands of the hinge. They appear to be well situated to effect an opening of the two domains when both are positively charged. The ovotransferrin structure indicates that the space in which the lysine side chains reside is tightly packed so that the result of both NZ atoms becoming positively charged will be domain opening and not simply a rotation of the two side chains away from one another. Moreover, the Lys–Lys couple is an interdomain interaction that would serve to hold the protein domains together at pH values above ~6. Low pK_a values for either one or both of the lysines in question might result from their lack of solvent exposure in a relatively hydrophobic environment, being surrounded by Tyr 82, Tyr 92, and Tyr 191. There are precedents for lysine residues exhibiting low pK_a values. For example, the pK_a of one of the active site lysines in acetoacetate decarboxylase is 6.0 (Kokesh & Westheimer, 1971).

The short 2.3-Å interaction between the NZ atoms of Lys 209 and Lys 301 appears to be an example of a low-barrier hydrogen bond where the proton is bound equally to both nitrogen atoms (Hibbert & Emsley, 1990; Cleland, 1992). In such an interaction the pK_a values of the two participants are

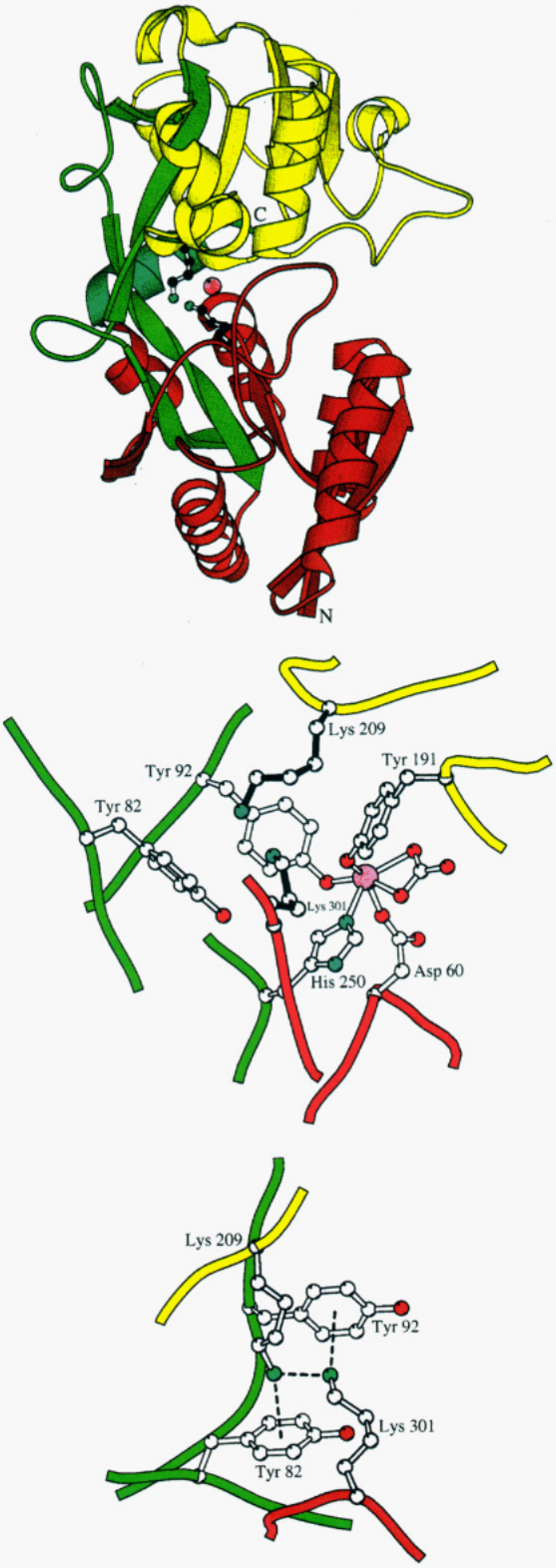

FIGURE 1: Three views of the monoferric N-terminal half-molecule of hen ovotransferrin from similar orientations. (A, top) Overall structure showing domain 1 (red) and domain 2 (yellow) with the interdomain cleft between them. The two β-strands that connect the domains and act as a hinge are in green (residues 74–100 and 224–255). The cleft opens to bulk solvent on the right side of the molecule as displayed. The cyan-colored helix (residues 321–332) is part of the connection to the absent C-terminal lobe. For clarity, only the Fe^{3+} ion (purple) and side chains of Lys 209 (top) and Lys 301 (bottom) are shown. Domain 1 consists of residues 1–90 and 249–320 while domain 2 consists of residues 91–248. Note that these residue ranges also incorporate the hinge region. (B, middle) Fe^{3+} binding site with Asp 60 and Lys 301 from domain 1, Tyr 191 and Lys 209 from domain 2, and Tyr 92 and His 250 from separate β-strands of the hinge. The bonds of the two lysine residues are in black while other colors are as above. (C, bottom) NZ atoms of Lys 209 and Lys 301 forming a low-barrier hydrogen bond with each other and amino–aromatic hydrogen bonds with the rings of Tyr 82 and Tyr 92. Hydrogen bonds are depicted by broken lines. Diagrams were prepared with MOLSCRIPT (Kraulis, 1991).

presumed to be similar (Cleland, 1992), and the hydrogen bond thus formed is very strong. Examples of other short hydrogen bonds in macromolecular structures, also presumed to be low-barrier hydrogen bonds (Cleland, 1992), include the glutamate to phosphinyl oxygen distances of 2.2–2.5 Å seen in six separate structures of carboxypeptidase (Kim & Lipscomb, 1990, 1991) and thermolysin (Tronrud et al., 1986; Holden et al., 1987) with phosphonate, phosphoramidate, or phosphonamidate inhibitors bound to the active site Zn^{2+}. The 2.3-Å distance we observe in the ovotransferrin structure appears to be comparable to these distances. One of the shortest N···N hydrogen bond distances observed in a small-molecule compound is the 2.54 Å in the structure of the monoprotonated derivative of the proton sponge 4,5-bis-(dimethylamino)phenanthrene (Staab & Saupe, 1988; Hibbert & Emsley, 1990).

Lys 209 and Lys 301 also appear to be stabilized by amino–aromatic hydrogen bonds (Burley & Petsko, 1986; Levitt & Perutz, 1988; Perutz, 1992) with neighboring tyrosine residues (Figure 1B,C). A hydrogen bond of this nature is formed between Lys 209–NZ and the π-electron cloud of Tyr 82 (NZ···ring–centroid = 3.8 Å) while Lys 301–NZ is similarly hydrogen bonded to Tyr 92 (NZ···ring–centroid = 3.5 Å). It is relevant that the position equivalent of Tyr 82 is conserved in the N-lobe of all members of the transferrin family listed in Table I, except for that of the hornworm. Tyr 92 is one of the ligands to Fe^{3+} and is presumably conserved if Fe^{3+} binding is to occur. The only possible classical hydrogen-bonding interactions involving the NZ atoms of Lys 209 and Lys 301 are Lys 209–NZ···Ser 303–OG = 3.7 Å, Lys 301–NZ···Tyr 82–OH = 3.2 Å, and Lys 301–NZ···Tyr 191–OH = 3.2 Å. In none of these cases, however, do the geometries suggest that any of these is a likely candidate for a hydrogen-bonding interaction.

Examination of the amino acid sequences of other transferrins (Table I) suggests that the Lys–Lys arrangement is a common occurrence in the N-lobe of the serum transferrins. Crystal structures are available for the rabbit and human serum transferrins. The structures of diferric rabbit serum transferrin (Bailey et al., 1988) and of its monoferric N-lobe (Sarra et al., 1990) reveal that the NZ atoms of Lys 206 and Lys 296 are ~3.0 Å apart, which suggests that a dilysine trigger could exist in the N-lobe of this protein although these two lysine residues have not been interpreted as such. The Asp ligand to Fe^{3+} in the rabbit serum structures has, however, been described as a trigger for domain closure (Grossmann et al., 1993). The human serum transferrin structure (Zuccola, 1993) is of the monoferric species, with the N-lobe in the open apo form, and therefore provides no information as to the existence of a Lys–Lys interaction. It would seem reasonable to suggest, however, that all N-lobe Lys–Lys groupings in closed Fe^{3+}-containing lobes of the serum transferrins will exist in the form of this pH-sensitive dilysine trigger. In the refined structure of the rabbit serum N-lobe (Sarra et al., 1990), Lys 206–NZ is directed at the ring of Tyr 85 (NZ···ring–centroid = 4.2 Å), and Lys 296–NZ is similarly directed at Tyr 95 (NZ···ring–centroid = 3.7 Å), suggesting amino–aromatic hydrogen-bonding interactions similar to those observed in ovotransferrin.

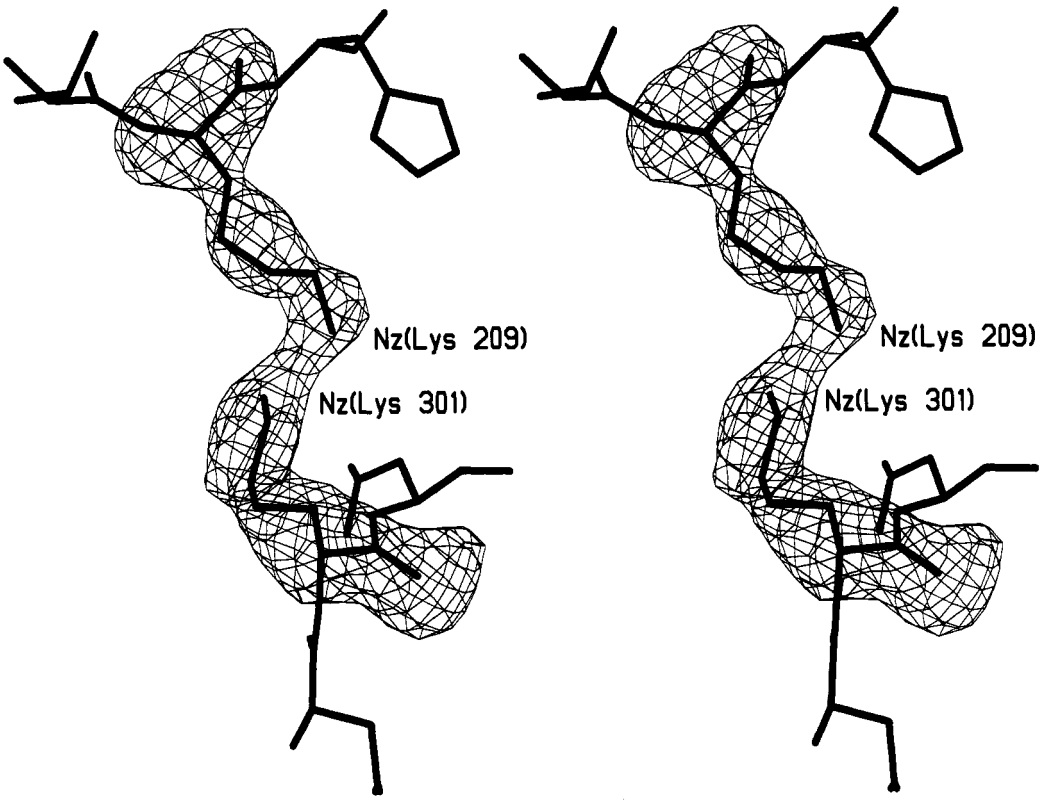

FIGURE 2: Stereo diagram of an electron density map with coefficients $|F_o|-|F_c|$ and phases calculated from a model after 30 cycles of refinement with TNT (Tronrud et al., 1988). All atoms of Lys 209 and Lys 301 were omitted from the refinement and map calculations. Contours are at the 3σ level. Substantial disorder in Lys 209 and Lys 301 seems unlikely in view of the relatively low values of their *B*-factors. The average *B*-factor for all atoms of Lys 209 and Lys 301, respectively, is 9.9 Å^2 and 7.9 Å^2 while corresponding values for the side-chain atoms are 10.6 Å^2 and 6.9 Å^2. For all atoms of the protein (which excludes residues 1–4, water molecules, Fe^{3+}, and CO_3^{2-}) the average *B*-factor is 27.0 Å^2. No alternative conformations in the electron density are apparent for either residue. Another difference map, calculated similarly, confirms bidentate binding by the synergistic CO_3^{2-} anion to Fe^{3+}.

Table I: Transferrin Sequence Alignment at the Possible Locations of pH-Sensitive Dilysine Triggers (Lys–Lys Couples)[a]

	N-lobe		C-lobe	
human serum transferrin[b]	Lys 206	Lys 296	Lys 534	Arg 632
rabbit serum transferrin[c]	Lys 206	Lys 296	Lys 531	Arg 628
horse serum transferrin	Lys 208	Lys 298	Lys 542	Arg 640
pig serum transferrin	Lys 210	Lys 300	Lys 543	Arg 641
rat serum transferrin[d]			Lys	Arg
Xenopus serum transferrin	Lys 207	Lys 282	Lys 534	Lys 632
hornworm serum transferrin	Lys 222	Lys 328	Ser 545	Asn 633
human melanotransferrin	Lys 209	Lys 307	Arg 555	Lys 655
hen ovotransferrin[e]	Lys 209	Lys 301	Gln 541	Lys 638
human lactoferrin[f]	Arg 210	Lys 301	Lys 546	Asn 644
mouse lactoferrin	Arg 209	Lys 300	Lys 543	Asn 641
bovine lactoferrin	Lys 210	Lys 301	Lys 544	Asn 642

[a] Entries are grouped into the serum transferrins and lactoferrins. Sequences are from the SWISS-PROT database (Bairoch & Boeckmann, 1992) except where a crystal structure is available in which case the sequence numbering is according to the structure. Sequence alignments were carried out with the program PILEUP of the GCG package (Genetics Computer Group, 575 Science Drive, Madison, WI 53711, Version 7, April 1991). Initial alignment was from Metz-Boutigue et al. (1984). [b] Crystal structure available; sequence from Zuccola (1993). [c] Crystal structure available; sequence from Bailey et al. (1988). [d] Partial sequence available. [e] This work with sequence from Jeltsch et al. (1987). [f] Crystal structure available; sequence from Anderson et al. (1989).

The Lys–Arg arrangement (Lys 534 and Arg 632) (Table I) in the C-lobe of the human transferrin structure (Zuccola, 1993) exists such that the shortest distance between these two residues is Lys 534–NZ···Arg 632–NH2 = 4.2 Å. The carboxylate group of the side chain of Asp 634 provides an oxygen atom that bridges both residues by hydrogen-bonding interactions where Lys 534–NZ···Asp 634–OD2 = 3.4 Å and Arg 632–NH2···Asp 634–OD2 = 3.3 Å. The position equivalent of Asp 634 is conserved in all serum transferrin C-lobes bearing the Lys–Arg arrangement, providing evidence for a common structural motif for this Lys–Asp–Arg triple in the C-lobes of these proteins. In the C-lobe of the unrefined diferric rabbit serum structure (Bailey et al., 1988) the disposition of Lys 531, Asp 630, and Arg 628 is similar to that of human transferrin.

The C-lobe Lys–Asp–Arg triple might represent a second type of pH-sensitive triggering mechanism for opening the transferrin domains. The two hydrogen bonds involving the aspartic acid residue that bridge the lysine and arginine residues would be broken or weakened at pH values near the pK_a that results in neutral aspartic acid. This could, in turn, lead to an opening of the C-lobe domains due to the opposed positive charges of the lysine and arginine residues. Glutamic and aspartic acid residues generally possess pK_a values of ~4.5 (Creighton, 1983; Fersht, 1985). It is also possible that breaking the salt bridge triple, when the aspartic acid residue becomes neutral, could facilitate domain opening simply by removing one of the interdomain interactions.

The pH-sensitive dilysine trigger (Lys–Lys couple) should effect N-lobe opening at a pH value somewhat higher than that at which the Lys–Asp–Arg triple would produce the same result in the C-lobe, although both would be operative below pH ~6. The presence of these groups in human serum transferrin provides an explanation for the *in vitro* experiments which demonstrate that, upon lowering the pH, the N-lobe (with the Lys–Lys couple) releases Fe^{3+} at pH ~5.7 while the C-lobe (with the Lys–Asp–Arg triple) retains Fe^{3+} until

pH ~4.8 (Princiotto & Zapolski, 1975; Lestas, 1976; Baldwin et al., 1982).

Several of the serum transferrin entries in Table I might appear to be anomalous in that they do not display the common arrangement of the Lys–Lys couple in the N-lobe with the Lys–Asp–Arg triple in the C-lobe. *Xenopus* transferrin, however, is missing the aspartic acid ligand in the N-lobe, and although it should bind Fe^{3+}, the lobe will likely not close completely (Lindley et al., 1993; Grossmann et al., 1993). The protein then presumably requires the dilysine trigger in the C-lobe if it is to function as a transferrin. Hornworm transferrin and melanotransferrin also bind only one Fe^{3+} ion (Bartfeld & Law, 1990; Baker et al., 1992), and Table I is consistent with this being in the N-lobe. Hen ovotransferrin and hen serum transferrin are derived from the same gene and differ only in their attached carbohydrate (Thibodeau et al., 1978). The protein would then have to serve dual roles as an Fe^{3+} transporter and as an antimicrobial. In the first role it must be capable of binding and releasing Fe^{3+}, which would be effected by the dilysine trigger we have observed in its N-lobe. The C-lobe seems to be designed for the latter role since no pH-dependent Fe^{3+}-release mechanism would be possible in the absence of the C-lobe Lys–Asp–Arg triple.

Table I suggests that no dilysine trigger can exist in either lobe of human lactoferrin. The crystal structure of the diferric form of this protein (Anderson et al., 1989) confirms this and also reveals that, in the N-lobe, Arg 210 occupies a position similar to that of Lys 209 of ovotransferrin but that Lys 301 forms a salt bridge with Glu 216 and is ~5 Å from Arg 210. Since Glu 216 and Lys 301 are on opposite domains, this salt bridge would tend to hold the domains together (Anderson et al., 1989; Baker & Lindley, 1992). It is conceivable that, at pH values below the pK_a value of Glu 216, the salt bridge would break, releasing Lys 301 to interact with the positively charged Arg 210 to effect a parting of the two domains. As with the Lys–Asp–Arg triple, however, simply protonating Glu 216 and breaking the interdomain salt bridge might facilitate domain opening. Glu 215 of ovotransferrin occupies the same position as Glu 216 of lactoferrin, but rather than form a salt bridge with Glu 215, Lys 301 forms the Lys–Lys couple with Lys 209. In the lactoferrin C-lobe there is no hydrogen-bonding interaction between Lys 546 and Asn 644 (Table I).

That neither lobe of human lactoferrin possesses the Lys–Lys couple, or the Lys–Asp–Arg triple, is in accord with the assumption that the primary function of the protein is as an Fe^{3+}-chelating antimicrobial agent with no requirement for a pH-dependent Fe^{3+}-release mechanism. Overall, it appears that if the lactoferrin N-lobe does possess an Fe^{3+}-release mechanism, it will not take effect until pH values that give neutral glutamic acid and, at least in terms of opposed positive charges, that appear to be responsible for domain opening elsewhere, no triggering mechanism appears available in the lactoferrin C-lobe. These differences, relative to the serum transferrins, help explain the observation that lactoferrin retains Fe^{3+} to lower pH values than human serum transferrin (Montreuil et al., 1960). Indeed, a site-directed mutant of the human transferrin N-lobe, with Lys 206 substituted by Gln, releases Fe^{3+} at pH 4 rather than the pH ~5.7 of the wild-type protein (Woodworth et al., 1991). This mutation transforms the N-lobe from one bearing the Lys–Lys couple to one with the Gln–Lys arrangement that is found in the ovotransferrin C-lobe. In agreement with the observations presented here, the human transferrin N-lobe has been transformed from an Fe^{3+}-releasing transferrin-type lobe to an Fe^{3+}-chelating lactoferrin-type lobe.

A suggested mechanism for Fe^{3+} uptake by the transferrins (Lindley et al., 1993) initially involves binding of the synergistic CO_3^{2-} anion to the open apoprotein followed by Fe^{3+} binding. The structure of the Fe^{3+}-containing quarter-molecule of domain 2 from the duck ovotransferrin N-lobe (Lindley et al., 1993) shows that the two tyrosinate ligands, along with the synergistic anion, are sufficient for the binding of Fe^{3+} to domain 2. Lindley et al. (1993) have also suggested that one of the final steps of the uptake mechanism involves ligation of Fe^{3+} by Asp, which also serves to close the two domains. As noted above, the Asp ligand has been described as a trigger for closure of the two domains upon Fe^{3+} uptake (Grossmann et al., 1993). Mutation of this Asp residue to either Cys or Ser in the human transferrin N-lobe (Woodworth et al., 1991) results in a molecule that does not close completely upon Fe^{3+} uptake as shown by solution X-ray scattering studies (Grossmann et al., 1993). The pH-sensitive dilysine trigger we observe in the hen ovotransferrin N-lobe and the Fe^{3+}–Asp bond are both interdomain interactions that must be broken for domain opening to occur, permitting release of Fe^{3+}. They must also re-form upon Fe^{3+} uptake and subsequent domain closure. It would appear, therefore, that the like positive charges of the two lysine residues of the dilysine trigger are, at least in part, responsible for the opening of the two transferrin N-lobe domains while the interaction of Fe^{3+} and the aspartate ligand would appear to be responsible for domain closure.

Bovine lactoferrin appears to be an anomalous entry in Table I for which we have no explanation at this time. The N-lobe appears to be of the serum transferrin type (Lys–Lys couple) while the C-lobe is of the lactoferrin type (Lys–Asn arrangement).

Early experiments (Fletcher & Huehns, 1967) suggested that the two lobes of human serum transferrin possessed different Fe^{3+}-release properties *in vivo* which subsequent work has both supported and contradicted (Huebers & Finch, 1987; Huebers et al., 1984). Fletcher and Huehns (1967) surmised that one lobe might be engaged in Fe^{3+} transport and release with the other acting more as an Fe^{3+} storage site. Our current observations provide a structural explanation for the different Fe^{3+}-release behavior displayed *in vitro*, and below pH ~6, by the two lobes of this molecule. The current situation, however, with regard to its behavior *in vivo* remains unclear and is the subject of continuing research (Bali & Aisen, 1991).

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