Coordination Chemistry Reviews, 22 (1977) 1—36

HUMAN SEROTRANSFERRIN: STRUCTURE AND FUNCTION

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(Received 12 July 1976)

CONTENTS

ABBREVIATIONS

apoTRN	apotransferrin
ATP	adenosine triphosphate
CD	circular dichroism
EDDHA	ethylenediamine di(*o*-hydroxyphenylacetate)
EDTA	ethylenediaminetetraacetate
EPR	electron paramagnetic resonance
Fe-TRN	monoferric transferrin
Fe_2-TRN	diferric transferrin
Fe_A-TRN	monoferric transferrin (A-site)
Fe_B-TRN	monoferric transferrin (B-site)

Fe-OT	monoferric ovotransferrin in which iron predominantly occupies the *N*-terminal binding site
OT-Fe	monoferric ovotransferrin in which iron predominantly occupies the *C*-terminal binding site
Gal	galactose
GlcNAc	*N*-acetylglucosamine
Man	mannose
NANA	*N*-acetylneuraminic acid
NMR	nuclear magnetic resonance
NTA	nitrilotriacetate
ORD	optical rotatory dispersion

A. INTRODUCTION

(i) Physiological role of the transferrins

As early as the 17th century, iron was recognized as an essential element in human nutrition. The body of the average adult male contains approximately 4.0 g of iron, sixty-five percent of which exists as hemoglobin iron [1]. The remainder is mostly found in the body's iron stores in molecules of hemosiderin and ferritin, and to a much lesser extent in other iron proteins, including the cytochromes, transferrin and myoglobin [1,2]. Certain tissues have a high demand for iron. Prominent among these are bone marrow, which uses iron in the biosynthesis of hemoglobin, and the placenta, which passes iron from the mother to the developing fetus [3]. To meet the demands of hemoglobin synthesis, the daily turnover of plasma iron in the normal human is a rather large 35 mg day^{-1} necessitating a system to regulate and rapidly transport metabolic iron. Serum transferrin, also known as serotransferrin (a siderophilin), is an integral part of this system.

Serum transferrin, a β-globulin which reversibly binds two moles of iron, transports this essential metal between sites of absorption (e.g., intestinal mucosal cells), sites of utilization (e.g., bone marrow), sites of storage (e.g., the liver) and sites of hemoglobin degradation [3—9]. It is believed that transferrin determines the distribution of iron in the body [3,5]. Transferrin is also important in controlling the buildup of toxic amounts of excess iron [5]. In serum, transferrin is about 30% saturated with iron; as a consequence it has the capacity to take on or release iron as required by various tissues. This is dramatically illustrated by the fact that in the blood, iron bound to transferrin turns over approximately ten times in a 24 h period [3,5]. Serum levels of transferrin are typically in the range of 200—400 mg/100 ml [10].

The term transferrin applies to the class of proteins which, in addition to the serotransferrins, includes the ovotransferrins and the lactotransferrins. Ovotransferrin (conalbumin) is found in rather large quantities in egg white. Its physiological function is not known with certainty. Because of its large affinity for iron, it is an effective antimicrobial agent which could be impor-

tant in the protection of the developing chick embryo [11]. In addition, ovotransferrin can donate iron to hemoglobin-synthesizing chick embryo red blood cells [13].

Lactotransferrin (also known as lactoferrin and milk red protein) was originally isolated from human milk [14] but has since been found in various external secretions [15]. Recent evidence based on peptide fragmentation patterns and sequencing studies indicate that lactotransferrin and serotransferrin were derived from the same ancestral iron binding protein [16,17]. The role of lactoferrin is not firmly established, but like ovotransferrin, its bacteriostatic activity might be important in depriving microorganisms of essential trace metals [15].

There are many similarities as well as differences, some rather subtle, between the various transferrins. The literature in this area is rather extensive. The reader is directed to several other sources for details and additional references [16—23]. This report covers the literature through March 1976.

(ii) Serotransferrin, molecular properties

Human serotransferrin is a glycoprotein with a molecular weight of about 77,000 daltons [24] and consists of a single polypeptide chain [24,25] of approximately 630 amino acids [26—28] and two identical carbohydrate prosthetic groups with molecular weights of nominally 2400 daltons each [27,29—31]. There are no subunits. The protein is capable of binding two Fe^{3+} ions tightly but reversibly [8,17,19,32]. Both metal binding sites appear equivalent in their affinity for iron at pH > 7 [32]. A large binding constant is necessary to avoid the formation of highly insoluble $Fe(OH)_3$, which is the most stable form of iron under conditions of physiological pH and oxygen tension in the absence of chelating agents [33]. How transferrin can bind iron so tightly and yet readily release it to tissue upon demand is a major unanswered question in transferrin chemistry.

Transferrin also binds a wide variety of divalent and trivalent transition metal ions as well as several of the lanthanides: Cu^{2+}, Zn^{2+}, VO^{2+}, Cr^{3+}, Mn^{3+}, Co^{3+}, Ga^{3+}, Ho^{3+}, Er^{3+}, Tb^{3+}, Eu^{3+}, Nd^{3+}, Pr^{3+} [23,34—43] and Gd^{3+} [44]. Most of these metal ions have served as useful spectrochemical probes of the metal binding site. Whether transferrin also plays an important physiological role in the transport of trace metal besides iron is not known. It has recently been reported that transferrin promotes the uptake of radioactive ^{67}Ga (a tumor scanning agent) in cultured mouse tumor cells [36]. There is some evidence that orally administered Zn^{2+} is transported from the intestine to the liver via portal blood transferrin in rat [139].

It is surprising that, despite the affinity of transferrin for a large number of very different metal ions, it does not bind Fe^{2+}, or at best binds it only very weakly, based on ultraviolet difference spectroscopy, isotope exchange experiments, and kinetic studies [45—47]. Morgan has suggested that reduction

of bound iron may be an important step in the release of iron to reticulocytes (immature red blood cells) [48].

It is now fairly well established that in order for iron to bind to transferrin, a suitable anion must be present [49—52]. In physiological media, this obligatory anion is carbonate (or possibly bicarbonate). In the absence of carbonate, numerous other anions such as oxalate, nitrilotriacetate, thioglycolate, etc., can promote binding [33,51,53,54]. Inorganic anions are ineffective [53]. Both the anion and the metal bind cooperatively 1 : 1 with the protein; neither binds appreciably without the other. Anions which exhibit this cooperative behavior have been referred to as "synergistic" anions [51,53]. Thus, there are two metal and two anion binding sites. The evidence, to be discussed in detail later, suggests that the anion and the metal sites are in close proximity to one another.

The spatial relationship between the two metal sites on the transferrin molecule is of particular interest. Luk has examined the fluorescence quenching of Tb^{3+} ions by Fe^{3+} ions in molecules of Tb^{3+}, Fe^{3+}-transferrin [34]. The lack of any significant energy transfer between Tb^{3+} and Fe^{3+} indicates that the metal sites are far removed from one another with a distance $\geqslant 43$ Å. Diferric transferrin is a prolate ellipsoid [55—58] with major and minor axes of $a = 55.2$ Å and $b = 27.6$ Å [55]. This places the metal sites essentially at opposite ends of the molecule. In many respects, it appears that the two regions of the protein which bind the metal can be considered as independent domains [12,59] although there is some evidence for an interplay between sites [60]. The same can be said of ovotransferrin [12,120,121].

At the time of this writing, human serotransferrin has not been completely sequenced [16,20,25—28,59,61—68]. However, it is now evident that enough homology exists between different regions of the polypeptide chain to conclude that a doubling of an ancestral gene occurred sometime in the distant past [59]. Sequence studies on various peptide fragments of human transferrin reveal considerable variation from one half of the molecule to the other. The amino acid sequence only imperfectly repeats itself. There is similar evidence for a duplicated polypeptide chain in ovotransferrin [69].

Some properties of human serotransferrin are summarized in Table 1.

(iii) Scope of this review

This review will deal primarily with human serotransferrin, hereafter referred to as simply transferrin. Emphasis will be on the major developments in the past six years [3—164]. Lactotransferrin and ovotransferrin will be considered only to the extent that they have a direct bearing on the discussion of transferrin. Clinical studies and investigations of transferrin/membrane interactions will not be covered to any significant extent. These topics are discussed in a recent book on iron storage and transport [4]. Several other aspects of the chemistry of the transferrins have been reviewed elsewhere [18, 19,21,22,72,149,159,164].

Through the efforts of a number of research groups, a great deal has been

TABLE 1

Properties of human serotransferrin

Property	Ref.
Molecular weight: 77,000 daltons	24
Single polypeptide chain glycoprotein	24, 25
Number of amino acid residues: 627,631	28, 63
Two equivalent carbohydrate prosthetic groups	29—31
Binds two Fe^{3+} ions	8
Apparent binding constant for Fe^{3+}: 10^{24} [a]	32
Does not bind Fe^{2+}	46, 71
Molar extinct. coeff. (apoTRN): $\epsilon_{278} = 9.23 \times 10^4\ M^{-1}\ cm^{-1}$	34
Molar extinct. coeff. (Fe_2-TRN): $\epsilon_{470} = 2.5 \times 10^3\ M^{-1}\ cm^{-1}$ [b]	32
Dipole moment (apoTRN): 990 D	55
Dipole moment (Fe_2-TRN): 930 D	55
Prolate ellipsoid	55—57
Axes (apoTRN): a = 62.0 Å, b = 24.6 Å	55
Axes (Fe_2-TRN): a = 55.2 Å, b = 27.6 Å	55
Intrinsic viscosity (apoTRN): $[\eta] = 0.037—0.041$	24, 55, 58
Sedimentation coeff. (apoTRN): $S^0_{20,w} = 5.25, 5.31$ S	24, 58
Sedimentation coeff. (Fe_2-TRN): $S^0_{20,w} = 5.38$ S	58
Diffusion coeff. (apoTRN): $D_{20,w} = 5.31 \times 10^{-7}$	57, 58
Diffusion coeff. (Fe_2-TRN): $D_{20,w} = 5.75 \times 10^{-7}$	58
Partial specific volume: $\bar{\nu}(\phi) = 0.725$ ml g^{-1}	70
Hydrated volume (apoTRN): $V_e = 15.4 \times 10^{-20}\ cm^3$	55
Hydrated volume (Fe_2-TRN): $V_e = 16.9 \times 10^{-20}\ cm^3$	55
Serum levels: 200—400 mg per 100 ml	10

[a] Calculated using the true equilibrium constant $K \sim 10^3$ as given in ref. 32 and assuming pH = 7.5 and $[HCO_3^-] = 2 \times 10^{-2}$ M, i.e., the approximate conditions in blood. [b] Per iron.

learned about the transferrin molecule. A few of the old controversies have been resolved but some still remain. The exact nature of the metal site is still unknown but a picture, albeit somewhat unclear, is beginning to emerge. One can now construct a reasonable model of the metal site; however, the exact identity of all the ligands and their geometrical arrangement about the metal are not known. The question of the origin of the equivalence or non-equivalence of the metal sites is still with us. Progress has been made in understanding the structural role of the anion and its implication in the mechanism of iron transfer from transferrin to the reticulocyte. The ultraviolet, visible, Raman and EPR spectral properties of transferrin are understood to some degree. We will now consider each of these areas.

B. NON-EQUIVALENT METAL SITES

(i) Functional heterogeneity

In 1967 Fletcher and Huehns observed that 50% saturated transferrin was less effective than 100% saturated transferrin in donating iron to red cell pre-

cursors (reticulocytes) on a per iron basis [6]. (It is customary to speak of "percent iron saturation" since the random or near random affinity of iron for the two sites results in a mixture of monoferric and diferric transferrins [32,73].) Moreover, they found that the two sites differed functionally in their ability to release iron. One site, referred to as the A-site, was more effective than the other, the B-site. The various transferrin complexes were ordered according to their iron donating capability $Fe_AFe_BTRN > Fe_ATRN > Fe_BTRN$. Their conclusions were later supported by both in-vivo and in-vitro experiments of a number of other groups [74—80].

These important observations led Fletcher and Huehns to hypothesize that transferrin is more than a passive iron carrier. They suggested that transferrin plays a key role, not only in iron transport, but also in the regulation of absorption and distribution of iron in the body [5]. In their model, iron is taken up randomly by the two sites but is selectively released from one site or the other depending on the particular tissue; i.e., the iron distributed on the transferrin molecule does not form a uniform pool from which various tissues can draw. They proposed that both the A- and B-sites can take on iron from the stores or intestinal mucosal cells, whereas only the A-site delivers iron to marrow, placenta and other iron-requiring tissues. The B-site alone deposits its iron in the stores for future use or in intestinal mucosal cells for desorption. In this way, it is possible to envision a scheme whereby the iron requirements of various tissues are met and the correct distribution of iron in the body is maintained (see Fig. 1) [5]. A schematic diagram showing the relationship between different iron proteins and their distribution in various tissues is given by Crichton [2].

The work of Fletcher and Huehns spurred considerable interest in the nature of the functional heterogeneity of the two sites. It was not clear whether the difference was solely one of function (perhaps in the way that

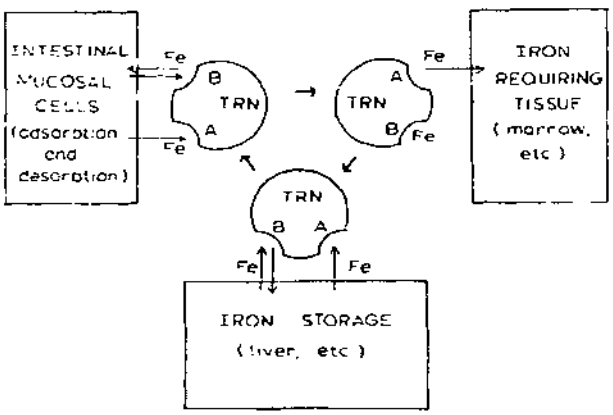


Fig. 1. Schematic diagram of the Fletcher—Huehns model for the role of transferrin in iron transport and regulation of iron metabolism. See text for explanation.

the protein bound to the receptor site of the reticulocyte) or an intrinsic difference in the metal ion coordination at the two sites. Numerous groups examined the protein for thermodynamic, spectroscopic and kinetic evidence of an intrinsic heterogeneity in the binding sites. These experiments will be discussed in the following sections after we first consider the nature of the functional heterogeneity in more detail.

Reticulocyte cells used for these studies are usually obtained by bleeding a rabbit regularly (phelbotomy) or by injecting the animal with phenylhydrazine; both procedures induce a high level of immature red cells in the blood. Recent experiments indicate that the phenylhydrazine procedure alters the receptor site on the reticulocyte membrane in some way [81]. Other experimental variables are also critical in studying transferrin—reticulocyte interactions [81].

Recently, Harris and Aisen in rather elegant studies have employed monoferric and diferric human transferrin obtained from isoelectric focusing techniques [75]. In this way, they could avoid working with mixtures containing all three transferrin species, Fe_ATRN, Fe_BTRN and Fe_AFe_BTRN, as was done in previous experiments employing transferrin of different degrees of iron saturation. Isoelectric focusing is made possible by the fact that the protein gains a negative charge for each Fe^{3+} ion bound [32,73], viz.

$$Fe^{3+} + apoTRN^{-z} + HCO_3^- \rightarrow [Fe\text{-}TRN\text{—}HCO_3]^{-z-1} + 3H^+ \quad (1)$$

They were able to show that the rabbit reticulocyte, on a per iron basis, took up radioactive ^{59}Fe from diferric human transferrin more readily than from monoferric transferrin, a result consistent with the work of others [6, 79,80]. In addition, the difference in efficiency could be accounted for by the observation that ^{125}I labeled Fe_2-TRN displays stronger affinity for the reticulocyte membrane than Fe-TRN [75]. Apotransferrin shows only a weak affinity for the membrane [7].

A difference in binding strengths of monoferric, diferric and apotransferrin with the reticulocyte is reasonable since apoTRN, Fe-TRN and Fe_2-TRN exhibit different chromatographic behavior on DEAE cellulose [82]. The *surface* charge on the protein, which is probably conformational dependent, becomes more electropositive as iron is added. This change appears to be limited to Fe^{3+} as opposed to divalent metal ions [138]. Lane has suggested that charge differences may play a role in the regulation of iron binding to membrane receptor sites. Moreover, it is well established that the protein changes conformation as iron binds, becoming more spherical and increasing slightly in size [55,58,83]. Compare the elliptical axes a and b, and hydration volume, V_e, for apoTRN and Fe_2-TRN in Table 1. Iron binding markedly reduces the extent of hydrogen—tritium exchange [156]. Changes in the proton NMR spectrum upon iron binding have been interpreted in part as a conformational change [103]. Conformational states of transferrin have been discussed by Llinas [21].

The importance of the metal in transferrin-receptor site interactions is

particularly emphasized by the fact that metal saturated Cu^{2+}, Cr^{3+} and Fe^{3+} transferrins exhibit quite different affinities for reticulocytes [84]. Uptake of divalent metal ions, i.e. Cu^{2+}, Mn^{2+} and Zn^{2+}, occurs without evidence for a specific metal transfer process in which the protein binds to the membrane with subsequent release of the metal [138]. Cu_2-TRN rapidly dissociates during incubation and is taken up by the cells, despite the fact that copper transferrin does not bind significantly to the cell surface [138]. There are between 26,000 and 45,000 transferrin binding sites per cell [84].

The effects of proteolytic enzymes and neuraminidase on iron uptake by reticulocytes have been studied by Morgan and co-workers [158]. Their results indicate that the receptor site is protein in nature. It appears that transferrin enters the cell by endocytosis, a process by which particles too large to diffuse through the cell membrane are taken up.

Harris and Aisen [75] also re-examined the functional ability of each of the sites of Fe_2-TRN to donate iron by first saturating apoTRN with radioactive ^{59}Fe. The $^{59}Fe_2$-TRN was then incubated with reticulocytes until about 25% of the iron was released to the cells. The cells were removed by centrifugation and the transferrin brought back to full saturation with ^{55}Fe. If one site is more effective in donating its iron than the other, then at this point, this site should be enriched in ^{55}Fe. The saturated transferrin was then incubated with fresh reticulocytes and the $^{59}Fe/^{55}Fe$ ratio in the total iron consumed by cells determined. A ratio of 1 indicates no difference between sites. Conversely, a ratio <1 indicates that one site is functionally superior to the other. Their results are shown in Fig. 2 [75]. One can clearly see that site heterogeneity exists when human transferrin is incubated with rabbit reticulo-

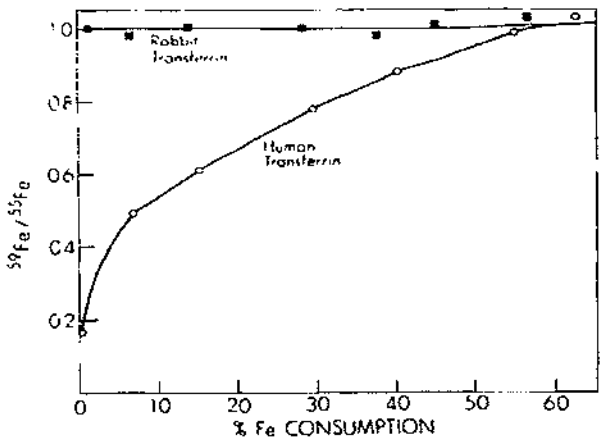


Fig. 2. Comparison of ^{59}Fe and ^{55}Fe uptake from doubly labeled human and rabbit transferrin by rabbit reticulocytes. See text for details of the experiment. (Reprinted with permission from D.C. Harris and P. Aisen, Biochemistry, 14 (1975) 262. Copyright by the American Chemical Society.)

cytes, particularly in the early stages of iron consumption. In contrast, the sites are homogeneous for rabbit transferrin incubated with rabbit reticulocytes. Furthermore, there is no difference in iron donating ability per iron bound to the protein for the rabbit/rabbit system in contrast with the results for the human/rabbit system discussed earlier.

This result raises the important question of species variability. Recently, Harris and Aisen [85] performed the critical experiment of using both human transferrin and reticulocytes; no functional heterogeneity was observed! This very significant finding suggests that some rethinking of the role of transferrin in regulation iron metabolism may be required. It should be noted, however, that their results are at variance with the in-vitro and in-vivo experiments with rats of Brown and co-workers [76—78,136].

Recent results indicate that neither ovotransferrin nor human lactotransferrin can donate iron to rabbit reticulocytes despite the similarities between these proteins and human serotransferrin [137]. It has been suggested that earlier observations of the uptake of iron from these proteins may have been due to the presence of non-specifically bound iron which was not removed from the protein prior to incubation with the reticulocytes (see ref. 137 for a discussion of this problem and leading references). On the other hand, it has been reported that the isolated iron binding fragments from ovotransferrin donate iron to rabbit reticulocytes [12].

(ii) Electron paramagnetic resonance spectra

Figure 3 shows the EPR spectrum of iron transferrin in frozen solution. The strong resonance at the effective g-value of 4.3 (~1500 G) and the associated weaker signal downfield at $g \sim 9.5$ (700 G) is found for many non-heme iron proteins and iron chelates having high spin Fe^{3+} in a rhombic crys-

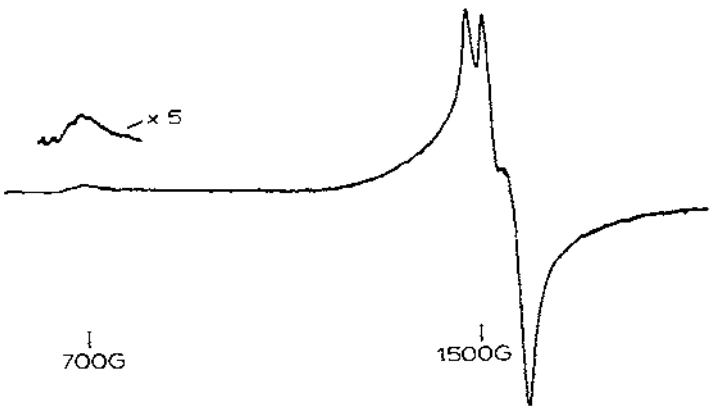


Fig. 3. Characteristic frozen (77 K) solution X-band EPR spectrum of 0.5 mM diferric transferrin in 0.01 M $NaHCO_3$, pH 8.5.

tal field environment [86—88]; however, the detailed features in the $g = 4.3$ region of Fig. 3 are unique to transferrin [32,89]. This characteristic spectrum has been a useful criterion for specific binding of iron to transferrin since small chelates exhibit $g = 4.3$ signals with different features [49,54]. Uncomplexed Fe^{3+} at physiological pH exists as a polymeric hydroxide which is difficult to detect by EPR [54].

This appropriate $S = \frac{5}{2}$ spin Hamiltonian for the rhombic case is given by

$$H = g\beta\vec{S} \cdot \vec{H} + D\left[S_z^2 - \frac{35}{12}\right] + E(S_x^2 - S_y^2) \qquad (2)$$

where D and E are the axial and rhombic components of the zero-field. H is the applied magnetic field and S_i ($i = x, y$ and z) is the electron spin operator in the molecular axis system. Here we have omitted quartic terms in the electron spin. For "S-state" ions lacking spin-orbit coupling, such as high spin Fe^{3+}, the g-factor is isotropic and approximates the free-electron value. It can be shown that a completely rhombic crystalline field, in which $E = D/3$ and $D \gtrsim h\nu$ (i.e., the zero field splitting exceeds the energy of the spectrometer), gives rise to a resonance with an effective g-value of 4.3 [87,88,90].

Full matrix calculations for various values of D and E have been used to obtain the principal resonance fields corresponding to the x, y and z axes as well as off axis extrema for rhombic iron spectra [88,90]. Off axis extrema produce additional lines in the spectra of powder (randomly oriented) samples. Although these calculations cannot account for the position of every line in the spectrum at both X-band (9.5 Hz) and Q-band (35 Hz) frequencies, the three component feature of the $g = 4.3$ resonance in Fig. 2 is predicted [88,90,91]. It appears that the essential features of the X-band powder spectrum can be simulated if one is willing to expend large amounts of computer time [91].

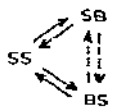
X-band and Q-band line position measurements yield $D = 0.27$ cm^{-1} and $E/D = 0.31$—0.32 for diferric transferrin [90]. Thermal depopulation of the $M_s = \pm\frac{3}{2}$ spin states (strong field notation) causes a loss in the EPR intensity of the $g = 4.3$ lines at low temperature [92]. Treatment of the intensity data with Boltzmann statistics yields $D = 0.32$ cm^{-1} and $E/D = 0.315$ [92]. The value of E/D near $\frac{1}{3}$ reflects the low symmetry environment of the metal ion. Comparable zero-field parameters are obtained for ovotransferrin and lactotransferrin indicating similar ligand fields for all the siderophilins [92].

Early investigations of the transferrin EPR spectrum as a function of iron saturation revealed no discernible difference between the two binding sites [54]. Aasa re-examined the spectrum as a function of microwave power and concluded that the $g = 4.3$ and $g = 9.5$ resonances consisted of overlapping lines of two rhombic type spectra [89]. However, the spectral differences attributable to the two sites are not very pronounced.

EPR spectra of monoferric and diferric transferrins obtained from isoelectric focusing are practically indistinguishable [60]. This result could be interpreted in one of three ways. (1) The two sites exhibit identical spectra;

this seems less likely in view of Aasa's results [89]. (2) The forms of monoferric transferrin (Fe_A-TRN and Fe_B-TRN) are present in equal amounts with each exhibiting its own spectrum, the sum of which is indistinguishable from that of Fe_AFe_BTRN. (3) The sites are "equivalent" but each exists in two conformations with each conformation exhibiting a unique spectrum. This latter interpretation is attractive in view of the results of Price and Gibson.

In 1972, Price and Gibson reported that sodium perchlorate had a pronounced effect on the EPR spectrum of iron transferrin [93]. With increasing $NaClO_4$ concentration in excess of 0.1 M, a broad $g = 4.3$ resonance (B) grows in at the expense of the sharp resonance (S) normally found for transferrin in the absence of perchlorate (Fig. 3). The perchlorate effect has also been observed with the EPR spectra of Cu^{2+}, VO^{2+} and Gd^{3+} transferrins [35,44,94]. Price and Gibson [93] attributed the observed spectral change to a perchlorate induced conformational change in the metal site; perchlorate, a so-called chaotropic agent, is known to alter the hydrogen bonding properties of aqueous solutions [150]. They proposed the following equilibrium scheme where S and B refer to the conformations responsible for the

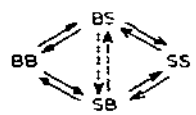


(3)

sharp and broad resonances respectively. In diferric transferrin, only one site (either site) can exist in the B conformation. Perchlorate shifts the equilibrium to the right.

Cannon and Chasteen [35] subsequently observed that the perchlorate effect is much more pronounced when transferrin is 50% iron saturated; i.e., more spectral broadening occurs. This can be explained by assuming that in the presence of perchlorate most of the monoferric transferrin exists in the B conformation whereas only one site has the B conformation in diferric transferrin. Accordingly, a larger percentage of the sites would be in the B conformation for a 50% saturated sample. Conformational differences in the two sites might be related to the heterogeneity in the donation of iron to rabbit reticulocytes by diferric transferrin as discussed earlier [93].

The perchlorate effect on ovotransferrin is opposite to that of transferrin, i.e., the sharp features increase with increasing perchlorate concentration [93]. Again, conformational changes can be evoked to explain the data. Perchlorate concentrations in excess of 1 M drive the equilibrium to the SS state of the



(4)

protein.

Additional insights into the conformation question of ovotransferrin have

been provided by the elegant work of Williams and co-workers in isolating the *C*-terminal and *N*-terminal iron binding fragments [12,95,96]. The fragments, each with a molecular weight around 35,000 daltons, specifically bind Zn^{2+}, Nd^{3+} and Pr^{3+} in addition to Fe^{3+} [95]. Moreover, they are capable of donating iron to reticulocytes as noted earlier [12,95]. Thus, the essential features of the intact protein, which are important to its function, remain relatively unaltered in the fragments. The sum of the spectra of the fragments is virtually identical to that of the native diferric protein. Each of the fragments exhibits B and S resonances (similar to those in Fig. 4) attributable to the simple equilibrium B ⇌ S, which shifts to the right with added perchlorate. At 0.7 M $NaClO_4$ the *C*-terminal fragment exhibits only sharp resonances which are indistinguishable from those of the *N*-terminal fragment at 1.4 M $NaClO_4$. Thus, the equilibrium positions of the two fragments are different but both are shifted toward the S conformation with increasing perchlorate concentration. These results indicate that the two halves of the ovotransferrin molecule are essentially independent conformational domains. Whether or not this is also the case for serotransferrin will have to await further experimentation.

EPR studies employing other metal ions to probe for differences in the sites of transferrin have met with varied success. EPR and magnetic susceptibility measurements with Co^{3+}, Cu^{2+} and Mn^{3+} have not demonstrated the presence of more than one type of specific binding [39,40]. In the case of Cr^{3+} transferrin, the EPR spectrum is a composite of resonances described by two

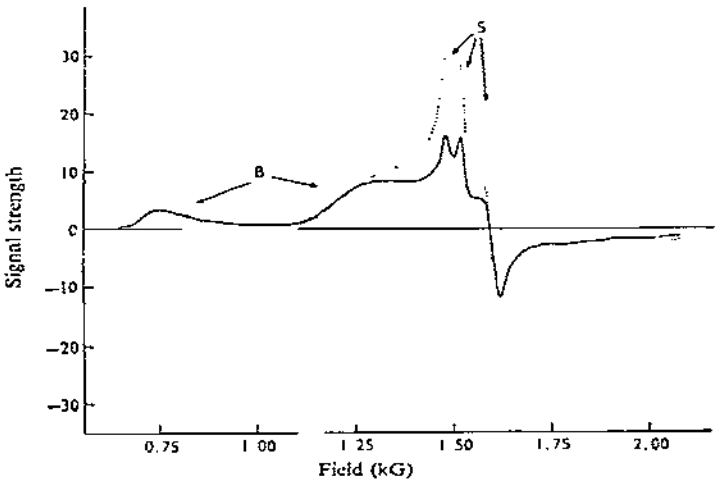


Fig. 4. Frozen solution X-band EPR spectra of monoferric hen ovotransferrins. 10% w/v in 0.1 M $NaHCO_3$ ——— Fe—OT; ····· OT—Fe; B, broad feature; S, sharp feature. (Reprinted with permission from R.M. Butterworth, J.F. Gibson and J. Williams, Biochem. J., 149 (1975) 559.) Consult this reference for similar spectra of the isolated iron binding fragments of ovotransferrin.

different Hamiltonians [39]. Lines attributed to the two Cr^{3+} species lose intensity sequentially as Fe^{3+} displaces the Cr^{3+} from the protein.

The vanadyl ion VO^{2+} has been the most successful spectroscopic probe in demonstrating heterogeneity in the binding sites [35,94]. The EPR spectrum of $(VO^{2+})_2$-TRN is shown in Fig. 5. The doubling of the resonance lines is due to the magnetic inequivalence of the metal ion environments, labeled A and B, in the transferrin molecule. For a discussion of vanadyl protein EPR spectra, the reader is directed to ref. 97. A detailed study of the effect of perchlorate and pH on the EPR spectrum indicates that the spectrum distinguishes between A and B conformations of the metal site in which the coordination geometry and/or identity of some of the ligands are different. At physiological pH the metal sites exist in the A and B conformations; however, when the pH is increased above 9, there is a gradual change from the A to the B conformation. This transformation is accompanied by the ionization of a single protein functional group, $pK_a = 10.0 \pm 0.1$, which does not coordinate to the metal. Tyrosine or arginine is a likely candidate.

Spectra of mixed Fe^{3+}—VO^{2+} transferrins suggest that A and B conformations possibly exist for the iron protein. Vanadyl transferrin has other properties similar to its iron counterpart [35,94,98]. It should be noted that the A and B labels employed here are arbitrary and do not necessarily correspond to those of the Fletcher—Huehns model (Fig. 1).

(iii) Thermodynamics of metal binding

Early work employing equilibrium dialysis of ferric transferrin against ethylenediaminetetraacetate (EDTA) indicated that the two binding con-

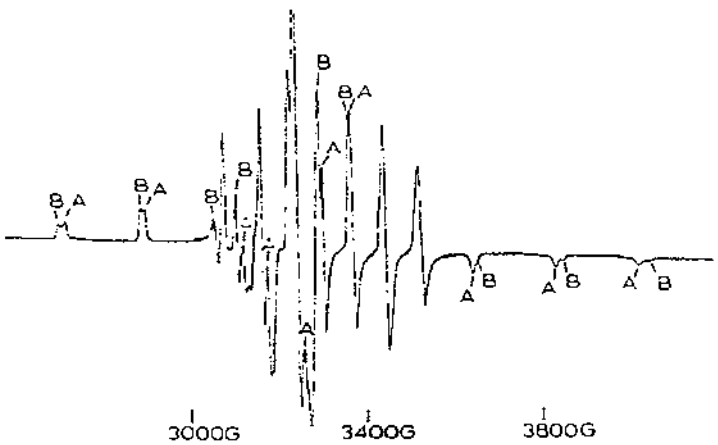


Fig. 5. Frozen solution (77 K) X-band EPR spectrum of divanadyl transferrin. 0.6 mM protein in 90% D_2O, pH = 8.4. A and B denote resonances due to two conformations of the metal sites.

stants for the two sites differ by a factor of 400 [99]. Subsequently, it was discovered that equilibrium had not been achieved, owing to the slow kinetics of the removal of Fe^{3+} from transferrin by EDTA [32]. More detailed work with citrate as well as EDTA as iron sequestering agents in competition with transferrin, demonstrated that, within experimental error, the binding of the first and second Fe^{3+} ions are equivalent, except for the statistical relationship, $K_1 = 4K_2$ [32,101]. This result is corroborated by moving boundary electrophoresis studies [73]. The binding of the first iron can be described by eqn. (1) for which $K_1 \sim 10^3$ M. Under conditions of pH and pCO_2 typical of blood (Table 1, footnote a) the apparent binding constant for the reaction

$$Fe^{3+} + \text{apoTRN} \rightleftharpoons Fe^{3+}\text{-TRN} \qquad (5)$$

is about 10^{24} [19].

Although there appears to be little difference in the affinity of Fe^{3+} for the two sites above pH 7, it has been found that in the pH range, 4.8—6.4, the dissociation of iron from the protein is biphasic, reflecting different binding strengths at the two sites [100]. At pH 5.8 only one Fe^{3+} is coordinated to the protein [100,157], a result corroborated by the observation that at pH 6.0 only 1.2 VO^{2+} ions bind to transferrin almost exclusively in the A conformation [35]. Fe^{3+} likewise binds at the A conformation at pH ~ 6 [98]. Furthermore, above pH 7, two VO^{2+} ions bind nearly equally to the A and B conformations with the B conformation slightly preferred. EPR studies with mixed VO^{2+}—Fe^{3+} complexes indicate that Fe^{3+} behaves similarly above pH 7 [35]. Since there are obviously structural differences between the metal sites as manifested in their different spectral properties, it is very surprising that they show essentially the same affinity for iron. Their binding constants cannot differ by more than 10 to 20%.

Binford and Foster have measured calorimetrically the enthalpies for the reaction of apotransferrin with Fe(III)—nitrilotriacetate (Fe-NTA) at pH 7.96 in the presence and absence of bicarbonate [102].

$$\text{apoTRN} + \text{Fe-NTA} + HCO_3^- \rightarrow \text{Fe-TRN—}HCO_3 + \text{NTA} \qquad (6)$$

$$\text{apoTRN} + \text{Fe-NTA} \rightarrow \text{Fe-TRN—NTA} \qquad (7)$$

For reaction (6) they obtained $\Delta H = -10.90 \pm 0.19$ kcal mol^{-1} for the process of bringing transferrin from 0 to 50% iron saturation compared to $\Delta H = -10.43 \pm 0.76$ kcal mol^{-1} for 50 to 100% saturation. Similarly for reaction (7), it was observed that $\Delta H = -4.54 \pm 0.05$ kcal mol^{-1} (0—50%) and -4.52 ± 0.08 kcal mol^{-1} (50—100%). Therefore, within experimental error the enthalpies for the binding of first and second equivalents of iron are the same for a given anion, NTA or HCO_3^-. Because of the near random nature of the metal binding, these ΔH values merely represent averages for the two sites and provide no information as to whether there is an intrinsic difference between the individual sites. The data do show, however, that the enthalpies of iron binding to monoferric versus apotransferrin are the same.

(iv) Sequence studies

The fact that the amino acid sequence is only imperfectly repeated in the second half of the molecule precludes the existence of identical metal ion environments in the two halves. Nevertheless, the local environments of the metal ions may be very similar and probably are. Evidence for this can be found in the recently reported sequence study by MacGillivray and Brew in which two peptide fragments were found to have a remarkably high degree of homology between them [59]. These authors point out that one would expect the homology in the peptide sequences making up the functionally important metal sites to be conserved even though the rest of the protein has undergone evolutionary changes. They propose that the highly conserved regions, positions 31 to 44, of the two fragments constitute the loci for the metal sites, viz.

	31											44
A	Tyr	Leu	Gly	Tyr	Glu	Tyr	Val	Thr	Ala	Ile	Arg	Asn	Leu	Arg
B	Tyr	Leu	Gly	Glu	Glu	Tyr	Val	Lys	Ala	Val	Gly	Asn	Leu	Arg

It is interesting that both fragments contain two tyrosines and an arginine (underscored). Numerous studies have shown that two (or possibly three) phenolate groups of tyrosyl residues bind the metal [23,34,37,41,94,103—107]. However, there is some evidence that the tyrosines involved in chelating iron are not localized in a small region of the polypeptide chain but instead are dispersed over a relatively large part of the peptide [160]. It has been suggested that part of the anion binding site may involve a positively charged group such as arginine [51,53].

The reader is referred to several literature sources for the amino acid sequences of other peptide fragments [16,26—28,59,61,63—68,108]. The amino acid composition of transferrin is given in Table 2.

The structure of the two carbohydrate groups, glycans I and II, has been completely determined [27,29—31,108,109]. They are identical branched structures consisting of 11 units each: 2 galactoses, 3 mannoses, 4 *N*-acetylglucosamines and 2 *N*-acetylneuramic acids as shown in Fig. 6 [31]. Both glycans are connected to asparagine of the peptide backbone by an asparaginyl-*N*-acetylglucosamine linkage.

(v) Kinetics of anion binding

Since there is a positive cooperativity between the anion and metal binding functions of the protein (see section C (i)), one might anticipate that heterogeneity in the metal sites would be reflected in the anion binding properties as well. This has in fact been observed.

Under conditions of physiological pH and ambient pCO_2, the exchange between bound and unbound carbonate is very slow with a half life $t_{1/2} \sim 20$

TABLE 2

Residue composition of human serotransferrin [a,b]

Residue	Number	Residue	Number
Lysine	57	Valine	40
Histidine	19	Methionine	7–8
Arginine	25	Isoleucine	14
Aspartic acid [c]	75	Leucine	56
Threonine	27	Tyrosine	21
Serine	37	Phenylalanine	26
Glutamic acid [d]	60	Galactose	4
Proline	30	Mannose	6
Glycine	46	*N*-acetylglucosamine	8
Alanine	53	*N*-acetylneuraminic acid	4
Cysteine	38		

[a] Taken from refs. 28 and 31. For other reported amino acid compositions see refs. 24 and 27. [b] Based on a molecular weight of 76,000. [c] Sum of aspartic acid and asparagine reported as aspartic acid. [d] Sum of glutamic acid and glutamine reported as glutamic acid.

days as followed by ^{14}C labeled carbonate. However, the rate of exchange is greatly accelerated by the presence of oxalate, citrate, nitrolotriacetate, thioglycolate or excess bicarbonate [60,135]. ATP, ADP and pyrophosphate likewise accelerate the exchange of carbonate while AMP, cyclic AMP and orthophosphate do not [134]. As might be anticipated, carbonate exchange in monoferric transferrin can be described by a simple first order process, first order in bound $^{14}CO_3^{2-}$. In contrast, interpretation of the exchange data for diferric transferrin requires two processes with half lives which fall on both sides of that for the monoferric protein. As an example, $t_{1/2}$ = 7.0 h for Fe-TRN compared to $t_{1/2}$ = 4.1 and 33 h for Fe_2-TRN when nitrilotriacetate is present (1 mM NTA, 0.062 mM protein, pH 7.5, 25°C) [60]. Similar results are obtained with the other catalytic anions or with excess unlabeled bicarbonate. This apparent site—site interaction is one of the few examples of cooperativity between the halves of the human serotransferrin molecule. The Fletcher—Huehns effect [5,6] may reside in the interplay between anion sites

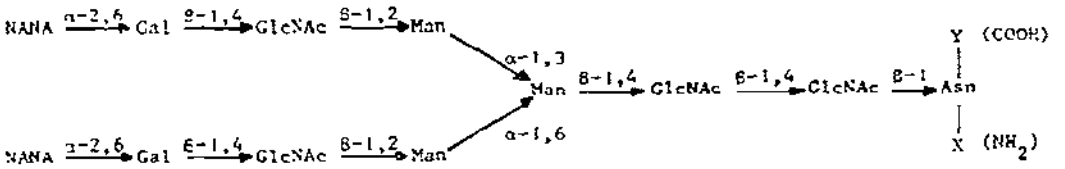


Fig. 6. Structure of the carbohydrate unit of the two glycopeptides GP-2 and GP-3 isolated from human serotransferrin. In glycopeptide GP-2, X = serine, Y = none; in glycopeptide GP-3, X = none, Y = lysine. (Reprinted with permission from G. Spik, B. Bayard, B. Fournet, G. Strecker, S. Gouquelet and J. Montreuil, FEBS Lett., 50 (1975) 296.)

[60]. The catalytic effect of small anions can be explained by the following mechanism.

$$\text{Fe-TRN—}{}^{14}\text{CO}_3 + \text{Anion} \rightarrow \text{Fe-TRN—Anion} + \text{H}{}^{14}\text{CO}_3^- \quad (8)$$

$$\text{Fe-TRN—Anion} + \text{HCO}_3^- \rightarrow \text{Fe-TRN—CO}_3 + \text{Anion} \quad (9)$$

Here, the steady state level of the Fe-TRN—Anion complex is very small relative to that of Fe-TRN—CO_3.

With dicobalt(III) transferrin, in the absence of catalytic anions, one site exchanges carbonate rapidly but the other does not. However, both anions exchange rapidly for the Zn(II), Ga(III) and Cu(II) transferrins [38]. The two anion sites in Fe_2-TRN—$(CO_3)_2$ have different susceptibilities toward displacement of carbonate by oxalate [110]. The initial rate of the reaction

$$\text{Fe-TRN—Anion} + \text{HCO}_3^- \rightarrow \text{Fe-TRN—HCO}_3 + \text{Anion} \quad (10)$$

has been measured for a variety of anions [53].

The carbonate exchange rates are different for the two sites in diferric ovotransferrin [13,111]. Metal free ovotransferrin binds only one anion specifically with an apparent association constant of 10^6 M^{-1} at pH 7 and 25°C [111].

(vi) Miscellaneous studies

Resonance Raman spectroscopy of copper and iron transferrins [41,112, 113], optical absorption spectra of mono- and diferric transferrins [60] and water proton NMR relaxation studies [43,114] have been incapable of distinguishing sites. Circular polarization of luminescence of mixed Ho^{3+}—Tb^{3+} and Fe^{3+}—Tb^{3+} complexes suggests that the two sites are equivalent in structure and conformation [42].

The two sites appear to have different size requirements. When rare earths are bound, the protein accepts two full Er^{3+} (0.881 Å) or Ho^{3+} (0.894 Å), slightly less than two Tb^{3+} (0.923 Å) or Eu^{3+} (0.950 Å) and only one Nd^{3+} (0.995 Å) or Pr^{3+} (1.013 Å). Ionic radii are given in parentheses [34].

Carbethoxylation of histidyl groups at or near the metal sites in serotransferrin or lactotransferrin results in the inability of the protein to bind iron at one site. Apparently the other site is buried and unavailable for chemical modification [115].

A study of the catabolism of human asialo-transferrin by guinea pig indicates a metabolic heterogeneity. This is apparently due to different affinities of the two glycopeptides comprising the protein for hepatic receptors when sialyl residues (*N*-acetylneuraminic acid) are removed from the two glycans [140].

(vii) Ovotransferrin (conalbumin)

The majority of the experiments with ovotransferrin point toward nonequivalent metal sites. Differential scanning calorimetry of Fe^{3+} and Al^{3+} ovo-

transferrins [116,117] and protein fluorescence [118] reveal differences but circular polarization of luminescence of Ho^{3+}—Tb^{3+} and Fe^{3+}—Tb^{3+} ovotransferrins does not [42]. Apparently the latter technique is not sufficiently sensitive. Mössbauer, optical difference and EPR spectroscopy of mixed Ga^{3+}—Fe^{3+} ovotransferrins indicate the presence of an "inner" and "outer" site [119]. The "inner" site must be occupied before binding can take place at the "outer" site. Circular dichroism and magnetocircular dichroism of Ga^{3+}—Fe^{3+} ovotransferrin complexes show that the protein sites are similar but not identical [37]. The possibility has been raised that the sites are equivalent before iron binding but are inequivalent afterwards [119]. Binding of iron at the two sites is anti-cooperative [120,121]. However, there is no difference in their ability to release iron to chick embryo red blood cells [13]. Similarly the *C*-terminal and *N*-terminal iron binding fragments donate iron equally rapidly to rabbit reticulocytes [12]. Other aspects of the metal sites in ovotransferrin have been discussed in sections (ii) and (vi) above.

C. ANION BINDING STUDIES

(i) The synergistic effect

Over the years, experiments designed to establish the cooperativity between metal and anion binding have led to conflicting results. The early work of Frankel-Conrat indicated that bicarbonate enhanced, but was not essential for color development of the iron ovotransferrin complex. This result suggested that the anion was not essential for binding of the metal [52]. The subsequent work of Warner and Weber failed to confirm this [125]. They concluded that the metal and bicarbonate bind 1 : 1 with the protein and that the anions bicarbonate or oxalate are essential for any color development. Aisen et al. employed EPR spectroscopy as a criterion for specific binding of Fe^{3+} to transferrin [54]. Without bicarbonate, the characteristic salmon pink color of the ferric transferrin complex was absent, but an EPR spectrum at $g \sim 4.3$ was still observed. However, the spectral details were significantly altered from that normally observed for iron transferrin. It was suggested that the spectrum was due to specifically bound iron in the absence of a bound anion. Price and Gibson later attributed the spectrum to a Fe^{3+}—citrate complex formed from a citrate impurity from the protein preparation [49]. It was found that citrate used in preparing apotransferrin binds tenaciously to the protein unless dialysis is carried out against perchlorate solutions. Price and Gibson also stated that bicarbonate was essential for specific Fe^{3+} binding to the protein. It was later suggested by Aisen et al. that the EPR signal was due to a Fe-TRN—citrate complex rather than a small iron citrate chelate [33]. However, Schlabach and Bates claim that citrate is ineffective in promoting specific iron binding [51,53]. Egyed, employing radioisotope exchange techniques, found that a binary Fe-TRN complex can be formed in the absence of anions [122]; this agrees with the results of Young and Perkins [110]. Raman

bands at 300 cm^{-1} and 360 cm^{-1} are only observed when bicarbonate is present and have been attributed to a Fe-TRN—HCO_3 ternary complex [71]. Oxidation of Fe^{2+} to Fe^{3+} is enhanced by apotransferrin only when CO_2 is present [45].

The disparity in the results of the various studies cited above is probably largely due to contamination of the solutions with bicarbonate or other anions. It is indeed difficult to prepare completely bicarbonate-free solutions and to avoid contamination of the solutions in subsequent manipulations from atmospheric CO_2 and other sources. It is presently known that over 20 different anions can promote specific iron binding [53]. Some of these anions are found in biological fluids and could be present in small amounts in protein preparations.

In order to resolve the anion question, Bates and Schlabach recently conducted an extensive investigation of the interactions between Fe^{3+}, transferrin and anions by preparing bicarbonate-free solutions by a variety of methods as a cross check of their procedures [50]. They employed over twenty-five anions [53]. Their results indicate that in the absence of anions, the specific Fe^{3+} sites cannot compete favorably with the formation of hydrolytic Fe^{3+} polymers and non-specific binding. This has been corroborated by the Mössbauer study of Tsang et al. demonstrating that without anions, iron binds non-specifically to the protein in the form of antiferromagnetic ferric hydroxide polymers [123].

Today, most workers probably agree that a strong cooperativity exists between the anion and metal binding functions of the protein. For several metal—transferrin complexes, the metal and bicarbonate are present in a 1 : 1 ratio (Table 3) [39,98,124,135].

In the absence of synergistic anions, transferrin binds copper(II) but not at the specific sites normally occupied by iron [40,45]. When bicarbonate is present, Cu^{2+} in excess of 2.0 per transferrin binds non-specifically at weaker binding sites [126]. A similar phenomenon is observed with Zn^{2+} binding to ovotransferrin [125]. Anions do not bind strongly to the protein in the absence of metal ions [39,111].

TABLE 3

Bicarbonate binding to metal derivatives of transferrin

Metal derivative	Bicarbonate/ metal ratio	Ref.
Fe^{3+}	0.94, 1.0	39, 135
Cu^{2+}	(1)	124
Cr^{3+}	1.09	39
Mn^{3+}	0.93	39
Co^{3+}	0.79	39
VO^{2+}	0.94	98

(ii) The anion binding site

The structural requirements for an anion to be synergistic are now fairly well established [33,49,51,53,54]. In order to be effective, an anion must have a carboxyl group and a second proximal electron-withdrawing functional group must be within 6.3 Å of the carboxylic acid group and be capable of adopting a "carbonate-like" configuration. Except for bicarbonate, oxalate forms [33,53,54]. Contrary to an earlier report [110], the inorganic ions SO_4^{2-}, NO_3^-, PO_4^{3-}, NO_2^-, SO_3^{2-} and BO_3^{3-} are also ineffective [53]. Neither ATP nor pyrophosphate can substitute for carbonate in the ternary complex [134]. Table 4 summarizes the organic anions which have been investigated. The synergistic anions are of the type $R-\overset{H}{\underset{L}{C}}CO_2^-$ in which L is a proximal electron-withdrawing functional group.

TABLE 4

Synergistic anions

Anion	Formula	Ternary complex [a] formed	Ref.
Carbonate	CO_3^{2-}	yes	49, 50, 53, 54
Oxalate	$^-O_2CCO_2^-$	yes	33, 53, 54, 110, 122
Malonate	$^-O_2CCH_2CO_2^-$	yes	33, 53, 54, 110, 122
Succinate	$^-O_2C(CH_2)_2CO_2^-$	no/yes	53, 110, 122
Maleate	$^-O_2CCHCHCO_2^-$	yes	53
Glyoxylate	$HCOCO_2^-$	yes	53
Pyruvate	$OC(CH_3)CO_2^-$	yes	53
α-ketoglutarate	$^-O_2C(CH_2)_2COCO_2^-$	yes	53
Ketomalonate	$^-O_2CCOCO_2^-$	yes	53
Acetoacetate	$CH_3COCH_2CO_2^-$	yes	53
Glycolate	$HOCH_2CO_2^-$	yes	53
Lactate	$HOCH(CH_3)CO_2^-$	yes	53
Phenyllactate	$HOCH(CH_2C_6H_5)CO_2^-$	yes	53
Methyllactate	$HOC(CH_3)_2CO_2^-$	no	53
Phenylglycolate	$HOCH(C_6H_5)CO_2^-$	yes	53
Malate	$^-O_2CCH_2CHOHCO_2^-$	yes	53
Gluconate	$HOCH_2(CHOH)_4CO_2^-$	yes	53
Salicylate	$o\text{-}C_6H_4(OH)CO_2^-$	yes	53
Citrate	$(^-O_2CCH_2)_2COHCO_2^-$	no/yes	33, 51, 53
Glycine	$H_2NCH_2CO_2^-$	yes	53
Phenylalanine	$H_2NCH(CH_2C_6H_5)CO_2^-$	yes	53
Thioglycolate	$HSCH_2CO_2^-$	yes	33, 53
Chloroacetate	$ClCH_2CO_2^-$	no	53
Nitrilotriacetate	$N(CH_2CO_2^-)_3$	yes	33, 53, 54
Ethylenediamine-tetraacetate	$(^-O_2CCH_2)_2N(CH_2)_2\text{-}N(CH_2CO_2^-)_2$	yes	53, 54

[a] Where conflicting results are reported, the most recent result is listed first.

By examining structure/binding relationships of these anions, Schlabach and Bates have concluded that the anion binding site is at least 3 Å deep, 6 Å wide and between 4 and 6 Å or more in length [53]. The site appears to be asymmetric and located near the surface of the protein [45]. The proximal group must be within 6.3 Å of the carboxylic acid group and be capable of adopting a "carbonate-like" configuration. Except for bicarbonate, oxalate forms the most stable iron-transferrin—anion ternary complex of all the other anions.

The spectral properties of the metal site are perturbed markedly when carbonate is replaced by other anions; this reflects the strong interaction between the metal and anion binding sites. Figure 7 shows the optical absorption spectra of a number of Fe_2-TRN—(anion)$_2$ complexes. There is reasonably good evidence that the visible absorption spectrum originates in a $p\pi \rightarrow d\pi$ ligand-to-metal charge transfer transition involving phenolate groups of coordinating tyrosine residues [37,41].

The EPR spectra of the complexes employing NTA, oxalate, malonate and thioglycolate as anions are shown in Fig. 8. The oxalate and malonate spectra indicate ligand environments intermediate between rhombic and axial symmetry (c.f. Figs. 3 and 8) [33]. High spin Fe^{3+} complexes of axial symmetry such as ferriheme are characterized by $g_\perp \simeq 6$ (1100 G) and $g_\parallel \simeq 2$ (3300 G) [127] as opposed to the $g \simeq 4.3$ (1500 G) for rhombic symmetry discussed earlier. The $g_\parallel = 2$ resonance for axial systems is usually weak; it is not ob-

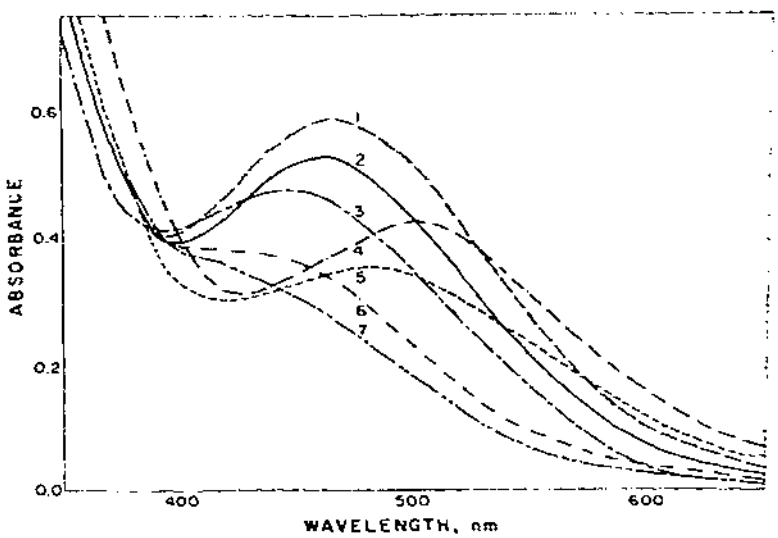


Fig. 7. Visible absorption spectra of Fe^{3+}—transferrin—anion complexes. The synergistic anions are: 1, nitrilotriacetate; 2, carbonate; 3, salicylate; 4, thioglycolate; 5, glycine; 6, glyoxylate; 7, glycolate. Ternary complex concentration of 0.20 mM with a 4 : 1 anion to iron ratio. (Reprinted with permission from M.R. Schlabach and G.W. Bates, J. Biol. Chem., 250 (1975) 2182.)

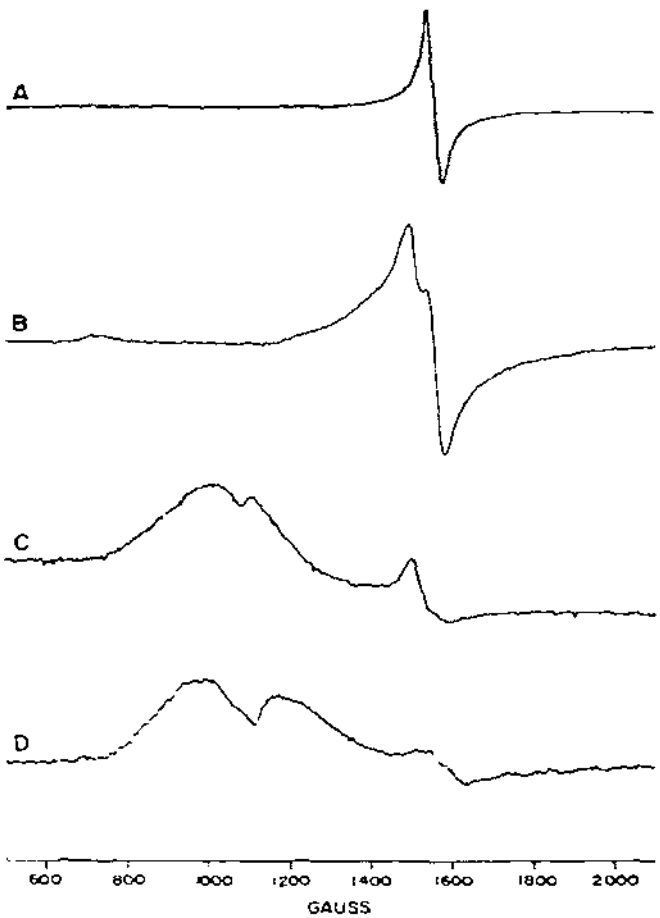


Fig. 8. Frozen solution (77 K) X-band EPR spectra of Fe^{3+}—transferrin—anion complexes employing the synergistic anions A. NTA, B. thioglycolate, C. malonate and D. oxalate. (Reprinted with permission from P. Aisen, R.A. Pinkowitz and A. Liebman, Ann. N.Y. Acad. Sci., 222 (1973) 337.)

served for transferrin. The order of increasing "axial symmetry" about the metal is: NTA < bicarbonate ~ thioglycolate < malonate ~ oxalate. The zero-field parameters likewise change with anion, $D = 0.32$ and $0.64\ cm^{-1}$ for bicarbonate and NTA respectively [92].

Table 5 summarizes the optical spectral data for these ternary complexes. While the wavelength of the absorption maxima are invariant (except for thioglycolate), it is interesting that the extinction coefficients correlate with the EPR spectra. If one assumes that the extinction coefficient is a measure of oscillator strength, then the data indicate that the charge transfer $p\pi \rightarrow d\pi$ transition loses intensity as the metal environment becomes more axial, i.e.,

TABLE 5

Optical spectral data for Fe_2-TRN-(anion)$_2$ complexes [a]

Anion	λ_{max}, (nm)	ϵ ($\times 10^{-3}$) (eq^{-1} cm^{-1}) [b]
Oxalate	465	1.60
Malonate	465	1.75
Thioglycolate	505	2.10
Bicarbonate	465	2.60
NTA	465	3.00

[a] From ref. 53. [b] Molar extinction coefficient per iron.

more highly symmetrical. This is a reasonable result. Earlier spectral data failed to reveal any relationship [54].

Optical spectra of the Cu^{2+}, Cr^{3+}, Mn^{3+} and Co^{3+} transferrins have also been investigated [39]. In all cases, an intense band is observed as well as lesser bands for some metals. The low intensity transitions are probably of *d—d* origin. The extinction coefficient of the primary band falls in the range 4×10^2 to 2.5×10^3 eq^{-1} cm^{-1} while its position varies from 405 to 465 nm. Not all metal ion derivatives of transferrin exhibit intense visible spectra, however. For example VO^{2+}—transferrin is colorless even though tyrosine is a ligand [35,94]. As for Fe^{3+}, the VO^{2+} EPR spectrum is a strong function of the anion bound.

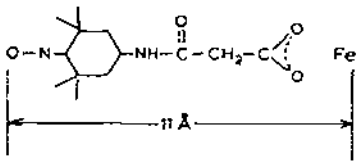
The dependence of the spectral properties on the anion could be a consequence of an allosteric effect induced by anion binding at a site removed from the metal. Alternatively, the anion could be directly ligated to the metal. Recent data discussed below support the latter interpretation.

The ^{13}C nuclear magnetic resonance line of Fe_2-TRN—($^{13}CO_3$)$_2$ is broadened beyond detection by the Fe^{3+} ($S = \frac{5}{2}$) paramagnetic center. On this basis, it has been established that the upper limit of the Fe^{3+}—^{13}C distance is only 9 Å which places the anion and metal binding sites in close proximity [38]. (NMR experiments with Zn^{2+} and Cu^{2+} are not possible because of rapid anion exchange.)

Results of EPR and Raman investigations, intended to show binding of the anion to the metal, have been inconclusive. Superhyperfine splittings from ^{13}C were not observed in the EPR spectrum of copper transferrin enriched in $^{13}CO_3^{2-}$ [38]. Carbonate does not contribute significantly to the resonance Raman spectrum of iron transferrin since no frequence shifts are observed when ^{18}O enriched carbonate is employed [41].

In a preliminary study, the loss in EPR signal intensity of a spin labeled anion has been used to calculate a metal—nitroxide distance of 11 Å [128]. This is consistent with direct linkage of the anion with the metal in the following way.

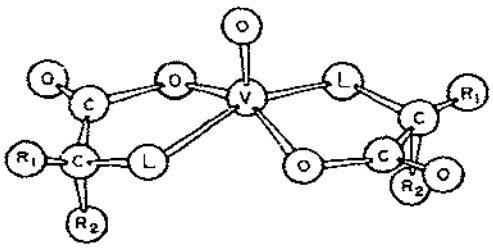
It seems likely that the anion is important in positioning the protein ligands to accept the metal ion, whatever the metal ion might be (see Table 3). It has



been suggested that the anion may neutralize positive charge repulsions in the region of the metal site by binding to a positively charged protonated ϵ-amino group of arginine [51].

Schlabach and Bates have proposed the interlocking sites model for metal and anion binding in which the anion bridges the sites by binding to the metal via the proximal functional group (Fig. 9) [53]. With this model, the anion dependence of the EPR and optical spectral properties can be understood in terms of changes in the proximal functional group. The mode of binding of the anion to the metal is opposite that inferred from the spin label study cited above.

Direct ligation of the anion to the metal through the proximal functional group appears to take place for the B-conformation of vanadyl transferrin [155]. For simple chelations of the type



the differences in their EPR parameters largely reflect variations in the proximal functional group L. The same can be said of the metal—protein—anion ternary complex if the ligand coordinates to the metal through the L group. Indeed, the $A_{\parallel}$ values for these two systems correlate nicely (Fig. 10).

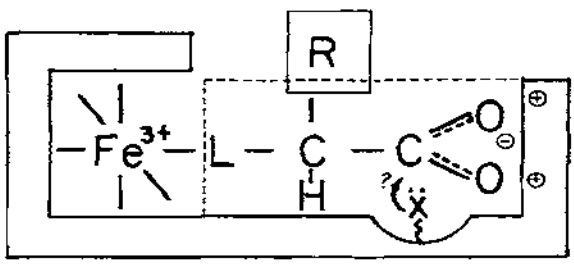


Fig. 9. Interlocking sites model for the binding of iron and synergistic anions to transferrin. — — — denotes restrictions on the size of the anion binding site based on molecular model studies. (Reprinted with permission from M.R. Schlabach and G.W. Bates, J. Biol. Chem., 250 (1975) 2182.)

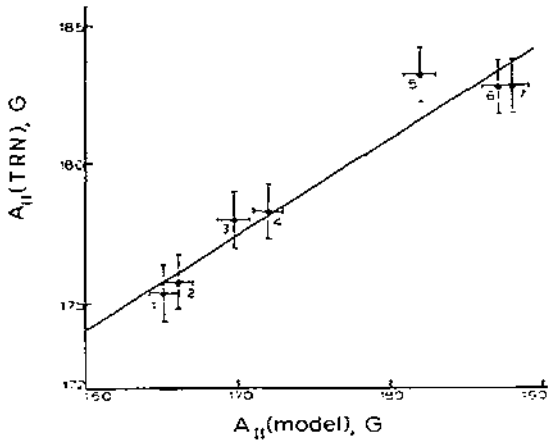


Fig. 10. Correlation of the parallel hyperfine splittings, $A_{\parallel}$, between VO^{2+}—transferrin—anion complexes and simple model complexes employing the anion as a ligand. Data for the VO^{2+}—transferrin—anion complexes are for the B conformation. The linear regression line with a correlation coefficient of 0.978 is shown. 1. thioglycolate 2. thiosalicylate 3. phenyllactate 4. lactate 5. salicylate 6. oxalate 7. malonate. (R.F. Campbell and N.D. Chasteen, submitted for publication.)

It is interesting that the positions of the A resonances of vanadyl transferrin are invariant with the anion bound; this suggests that the anion might not be coordinated to the metal in the A-conformation. However, anion binding is a requisite for metal binding to the protein. Appreciable binding in the A-conformation occurs only when "multicarboxylate" anions such as carbonate, oxalate, NTA and malonate are employed. This suggests that possibly two positively charged protein groups must be "neutralized" by the anion before the metal can bind in the A-conformation.

The ^{13}C resonance of diamagnetic Co^{3+} transferrin is 18 ppm downfield from free $H^{13}CO_3^-$ which suggests that the anion is bound as carbonate rather than bicarbonate. This is in accord with the early proposal that ovotransferrin binds CO_3^{2-} [125]. Recent stability constant data is best understood if one assumes that the dianion binds [111]. The mechanism of Fe^{2+} oxidation in the presence of transferrin is likewise consistent with bound carbonate [45].

To explain the fact that inorganic anions other than carbonate are ineffective, it has been suggested that the anion may become covalently linked to the protein by nucleophilic attack on the anion carboxylate carbon by a protein functional group, Ẍ, to form tetrahedral carbon (Fig. 9) [51,53].

(iii) Functional role for the anion

As noted in the Introduction, one of the fundamental questions in transferrin chemistry is the mechanism of iron release from the protein to tissue upon demand. Available evidence suggests that the anion plays a key role in this pro-

cess. It has been shown by two different groups that replacing carbonate by oxalate reduces the rate at which transferrin donates iron to the reticulocyte by some 65% [33,122,129]. The same is observed for the ovotransferrin and chick embryo red blood cells [13]. Malonate is more effective than oxalate but less so than carbonate [129].

It has been suggested that the residual activity of the Fe-TRN—oxalate is actually due to Fe-TRN—CO_3 formed from the displacement of oxalate by HCO_3^- present in the reticulocyte solutions [149]. Both the carbonate and oxalate ternary complexes show the same affinity for the membrane receptor site.

The rate of release of $H^{14}CO_3$ from transferrin is the same as the rate of iron incorporation into the reticulocyte, suggesting a cooperative mechanism in the cell for release of both Fe^{3+} and HCO_3^- [135]. The importance of the anion is further amplified by kinetic studies which reveal that the protein must first bind carbonate before sequestering the iron [45].

It has been proposed that transferrin binds to the receptor site on the membrane surface followed by cleavage of the bond of the metal—protein—anion ternary complex [13,33,122,129,135]. Presumably, the anion is released first followed by the metal. Apparently, oxalate is less susceptible to attack than carbonate. ATP may also play a key role in the release of iron [134].

The release of CO_3^{2-} is catalyzed by CO_2 and H_3O^+ [111]. If a proton transfer from a functional group (such as arginine) on the reticulocyte to the anion is responsible for the release of iron, one could explain the difference in iron-donating capability of the two metal sites on the basis of differing susceptibilities of the anion sites to protonation. A difference in the intrinsic pK's of the anion in the two sites is consistent with the observation that the two metal sites show different affinities for Fe^{3+} or VO^{2+} below pH 7 [35,100]. The lower rate of iron release when carbonate is replaced by oxalate could be explained on the basis of the lower pK's for the latter anion [13]. In fact, two VO^{2+} ions bind the protein at pH 6.0 when oxalate is the anion compared to only one when carbonate is the anion [155]. The loss of binding at the B conformation of vanadyl transferrin upon lowering the pH below 7 is coupled to the protonation of a functional group with an apparent pK = 6.6 [98]. This is possibly the anion itself.

D. IDENTITY OF THE LIGANDS

Ultraviolet difference spectra of various metallotransferrins, with apotransferrin as a blank, display two prominent bands at approximately 245 nm and 295 nm, which have been attributed to binding of ionized phenolate groups of tyrosine to the metal. One or both of these bands have been observed for Fe^{3+}, Mn^{3+}, Cu^{2+}, Co^{3+}, VO^{2+}, Ga^{3+} and Tb^{3+} siderophilins [23,39,94,103—106]. Based on the change in molar absorptivity attending the ionization of model compounds such as *N*-acetyltyrosine, the number of coordinating tyrosines has been estimated to be two per metal [94,103,104,106]. The validity

of this procedure has been challenged because the effect of the coordinating metal ion on the molar extinction coefficient has not been taken into account [23]. Fluorescent lifetime measurements on lanthanide transferrin complexes indicate that only two tyrosines coordinate to the metal [34]. However, this determination is likewise based on model compounds which do not involve a coordinating metal ion. Six additional tyrosines are titrated with base in apotransferrin compared to diferric transferrin, implicating three tyrosines in each metal site. Chemical modification of ovotransferrin by iodination suggests two tyrosines per site, in agreement with the spectroscopic procedures [107]. On the other hand, nitration of tyrosine suggests three per iron in transferrin [160]. The discrepancy in the number of tyrosines as determined by these various procedures could possibly arise from the presence of an extra tyrosine in the region of one metal site, as suggested by sequencing studies (compare chains A and B in section B (iv)). Perturbations in the proton magnetic resonance of the siderophilins as a result of binding Ga^{3+} or Fe^{3+} also indicate involvement of tyrosine in metal complexation [103].

The intense visible absorption band at 470 nm for diferric transferrin has been compared with that of the model iron complex Fe(III)EDDHA, in which there are two coordinating phenolate groups [41]. For complexes of this type, the intensity and position of the visible band are sensitive to the number of coordinating phenolate groups in a predictable way. The authors conclude that in the protein complex, there are two tyrosines bound to the metal.

The resonance Raman spectrum of iron transferrin shows resonance-enhanced bands at 1604, 1504, 1284 and 1173 cm^{-1} which have been assigned as various phenolate vibrational modes (as opposed to metal—ligand vibrations) [41,113]. Previous assignment [112,113] of some of the bands to imidazole vibrations appears to be incorrect [41]. Model compounds of copper and iron have been used to aid in the assignments of the Raman spectra of the corresponding protein complexes [41,130].

It has been suggested that in addition to tyrosine, typtophan might also contribute to the ultraviolet difference spectrum of transferrin [106]. Absorption and emission spectroscopic properties of colored Fe^{3+}, Cr^{2+}, Cu^{2+} and Mn^{3+} ovotransferrins and colorless Zn^{2+}, Co^{2+}, Ni^{2+} and Cd^{2+} ovotransferrins reveal that two tryptophan residues are bound to the metal or in close proximity in the colored complexes [105]. Apparently, tryptophan does not "participate" in the metal site of all metallo—ovotransferrins because of different coordinating properties of each metal. In iron-saturated ovotransferrin, two tryptophan residues per protein molecule are protected by the iron [107] from oxidative modification.

Carboxymethylation of histidine groups with bromoacetic acid results in 11—12 groups modified in the first 48 h. An additional 2—4 groups are modified over a 12 day period during which iron binding is lost, implicating 1 or 2 imidazoles per iron site [131]. Histidines have also been labeled with diethylpyrocarbonate which has the advantage of reacting rapidly in approximately $1\frac{1}{2}$ h [115]. The results indicate that up to 3 histidines are involved in binding

the site. The EPR spectrum of copper transferrin in the presence of bicarbonate shows a three component splitting in the parallel copper hyperfine lines; this splitting has been attributed to a superhyperfine interaction from a coordinating nitrogen ligand, possibly imidazole [40]. It should be noted, however, that iron and copper transferrins are not completely analogous, in that two protons are released per Cu^{2+} bound compared to nominally three for iron [32,73,125,132].

Water proton nuclear magnetic resonance relaxation enhancement measurements show that one water molecule is bound 2 Å from iron and another 5 Å distant [114,161]. Likewise, one exchangeable water molecule is present in the first coordination sphere of copper transferrin [43]. Water has been implicated in the first coordination sphere of vanadyl transferrin [98]. The vanadium hyperfine coupling constants and g-values from the EPR spectrum suggest that most of the equatorial ligands are oxygen donors [94].

The circular dichroism (CD) of transferrin and ovotransferrin and their copper complexes indicate differences in metal binding to the two proteins [126]. Their iron EPR spectra likewise reflect differences [93]. Therefore, conclusions regarding the ligands or coordination geometry for one protein may not necessarily apply to the other.

The CD spectrum of transferrin or ovotransferrin does not change significantly upon binding of Cu^{2+}, which suggests that the metal binds to an essentially preformed binding site and causes no major change in the backbone or surface conformation of the protein [126]. The protein has low helix structure (~20%) and contributions from antiparallel β-pleated sheet to the total structure [126,133]. The CD and ORD properties of the siderophilins have been reviewed elsewhere [18,21,126].

With the available data, it is not possible to construct an explicit model for the metal site in transferrin. The studies cited here indicate a maximum of three tyrosines, three histidines, two tryptophans, a water molecule and carbonate as ligands. Clearly all these ligands cannot be bonded to the metal, for this would result in ten-coordinate iron. The possibility has been raised that the metal site may be seven-coordinate [40]. Iron compounds of this type are known.

The difficulty in assessing the number of coordinating ligands from chemical modification studies lies in the fact that it is not generally possible to distinguish between functional groups which are merely protected by the metal from those which are directly bonded to it. Moreover, loss of iron bonding due to modification of particular residues may simply reflect a change in protein conformation at the metal site and have nothing to do with bonding of the modified residues to the metal. Substitute transition metal ions may not accurately probe all the ligands which bind iron. The fact that some metallotransferrins are intensely colored, while others are not, suggests that this may be the case [35,105].

Despite these limitations, a reasonable view of the metal site might include two tyrosines, a water molecule, carbonate and one or two histidines as lig-

ands. One or two tryptophans could also be coordinated (or nearby) to give a six or seven-coordinate metal site. One possibility is that there are several states of the metal site which involve different geometries and ligands. In other words, the metal site may be ligand rich, capable of existing in several states. The observation of three "ligand environments" about the VO^{2+} ion due to different metal site conformations as the pH is raised from 6.0 to 10.7 might be construed as evidence for this [98]. The same could be said of the three or four nitrogen ligands evidenced in the EPR spectrum of "non-specifically" bound copper compared to one nitrogen ligand for specifically bound copper [40,43,45], and the involvement of tryptophan in the binding of some metals and not others [105]. Moreover, the fact that the protein binds very diverse metal ions (see Introduction) suggests that there is considerable flexibility in the metal site.

E. DELIVERY OF IRON TO APOTRANSFERRIN

Metal chelates are known to play a fundamental role in trace metal metabolism and transport in numerous biological systems [141]. Model studies of the delivery of iron to apotransferrin by metal chelates are important in that they may provide insights into the mechanisms of biological metal ion exchange reactions. This was the motivation for much of the early work on the delivery of iron to transferrin.

Some small chelates are more effective than ionic iron in delivering the metal to transferrin. The times required to half saturate transferrin using the Fe^{3+} complexes of NTA, citrate and EDTA are 3 s, 8 h and 4 days respectively [142]. The ease with which these chelating agents remove iron from the protein follows the reverse order of their ability to donate iron [143]. Interestingly, the kinetics of iron delivery to transferrin do not correlate with the stabilities of the metal complexes [143]. This can be explained on the basis of the proposed mechanisms of iron delivery to these chelates [142—144]. Intermediates of the type Fe^{3+}-TRN—chelate appear to be important. Also, ligand exchange reactions may be rate limiting.

The slow reaction of Fe—EDTA with apoTRN may be due to the necessity of forming a Fe—EDTA* species, present in small concentration at equilibrium, in which the chelate has partially unfolded from about the metal, thus permitting attack by the amino acid ligands of the protein [143]. The polymeric character of iron citrate can account for its low reactivity [142].

Stopped-flow measurements indicate that the reaction of Fe-NTA with apoTRN is biphasic. The following mechanism has been proposed [144].

$$\text{Fe-NTA} + HCO_3^- + \text{apoTRN} \rightarrow \underset{\text{(intermediate)}}{\text{Fe-NTA—TRN—}HCO_3} \qquad (11)$$

$$\underset{\text{(intermediate)}}{\text{Fe-NTA—TRN—}HCO_3^-} \rightarrow \text{Fe-TRN—}HCO_3 + \text{NTA} \qquad (12)$$

The first step is complete after a few tenths of a second. The second step requires approximately 10 s. Evidently the intermediate is unlike transferrin complexes in which the NTA is merely substituted for bicarbonate. Use of Fe-NTA is a preferred way of introducing iron stoichiometrically into apotransferrin.

For several metal complexes, donation of iron to transferrin follows saturation kinetics with respect to bicarbonate; this suggests that apoTRN—CO_3^{2-} is the agent which is formed to complex the metal [45]. The enthalpy for the reaction of Fe-NTA with apoTRN has been measured (see section B (iii)) [102].

Addition of ferric salts to solutions of apotransferrin above pH 5 yields transferrin complexes of stoichiometry less than 2 : 1 because of competitive hydrolysis of the iron [147]. Moreover, use of $FeCl_3$ or $Fe(NH_4)_2(SO_4)_2$, especially the former, results in some non-specifically bound iron [81]. Titration of apoTRN with ionic Fe^{3+} gives no clear spectrophotometric endpoint at 470 nm [152]. Ionic Fe^{3+} can be bound stoichiometrically to the protein providing that it is introduced at low pH and subsequently the pH of the metal—protein solution slowly raised. In contrast, the reaction with Fe^{2+} is smooth and stoichiometric above pH 6.5 and is enhanced by oxidizing agents [147]. On the basis of kinetic evidence, the mechanism of oxidation of Fe^{2+} by molecular oxygen in the presence of apoTRN has been proposed [45], viz.

$$\text{apoTRN} + HCO_3^- \rightleftharpoons \text{apoTRN—}CO_3^{2-} + H^+ \quad (13)$$

$$\text{apoTRN—}CO_3^{2-} + Fe^{2+} \rightleftharpoons Fe^{2+}\text{-TRN—}CO_3^{2-} \quad (14)$$

$$Fe^{2+}\text{-TRN—}CO_3^{2-} \xrightarrow{O_2} Fe^{3+}\text{-TRN—}CO_3^{2-} \quad (15)$$

Frieden and co-workers have studied the migration of iron from phosvitin to transferrin and its possible significance in iron metabolism [146]. The transfer is rapid with a half-life $t_{1/2}$ = 10 min. Phosvitin may mediate the transfer of iron from ferritin to transferrin in hen plasma. A mechanistic scheme has been proposed for this [146].

The exchange of iron between transferrin and ferritin has been reviewed by Crichton [2].

F. CONCLUSION

Our knowledge of transferrin is currently in a state of flux. Various ligands have been implicated in the bonding of the metal, but the exact number and identity remain a matter of some controversy. Indeed, there is probably more than one configuration about the metal, in which one or more of the ligands is different. The delineation of the ligands in the region of the metal site may have to await an X-ray crystal structure determination and the complete amino acid sequence. Preliminary X-ray crystallographic data have been

reported [151]. Crystals of transferrin are readily obtained [152].

The spatial and functional relationship between the metal and anion binding sites is partially understood, at least in broad terms. There is little doubt that the anion is bound to metal in at least one conformational state of the metal site. A hypothesis for the anion function has been formulated in which protonation of the anions triggers the release of the metal to iron-requiring tissues. However, the importance of protein conformation on the state of the metal site must also be taken into account. It seems likely that a change in protein conformation upon binding to the receptor site as well as protonation of the anion is important in the transfer of iron to the cell. Relatively little is known about the structure of the transferrin—anion receptor site complex.

The recent discovery of the involvement of transferrin in zinc transport (see Introduction), prompts one to speculate about the possibility that this protein is a carrier for some essential trace metals other than iron. Possibilities are Co^{2+}, Cr^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Mo^{4+} and V^{4+} [153]. Zinc, which is the second most abundant transition metal in the body (~2.6 g per 70 kg man) is an integral part of some 70 metalloproteins [154]. It is possible that a protein, such as transferrin, plays some role in the distribution of metabolic zinc. Transferrin has also been implicated in the transport of trivalent chromium [164]. The establishment of transferrin as a general carrier of trace metals would give added significance to the many studies of various metal derivatives of this protein and open up new avenues of transferrin research.

ACKNOWLEDGEMENT

This work was supported by the National Institute of General Medical Sciences, Grant GM 20194-04.

ADDENDUM

Additional evidence for the absorption and transport of zinc by transferrin has appeared [165]. Studies of iron uptake by rabbit reticulocytes from ^{59}Fe transferrin labeled at different pH values suggests a site heterogeneity in the release of iron [137,166]. The metal site which binds iron at low pH (~5.8) is the one which does not readily release its iron to reticulocytes. Selectivity in the release of iron to reticulocytes, liver, and bone marrow, and in the uptake of iron by transferrin from intestinal mucosa has been reported [167]. Other studies have also implicated ATP in the uptake of iron by erythropoietic cells [168]. A transferrin receptor site glycoprotein of molecular weight ~130,000 daltons has been isolated from reticulocytes. There are approximately 60,000 such sites per reticulocyte [169]. Phospholipase A inhibits uptake of Fe by rabbit reticulocytes; this effect is related to the phospholipid content of the membrane and suggests that phospholipids play a role in iron transport [170]. Iron uptake by rat liver mitochondria proceeds by two mech-

anisms, one energy dependent and the other energy independent [171]. Human serum transferrin has been fragmented using N-bromosuccinimide [172]. However, this procedure does not appear as useful as the cyanogen bromide method. An iron binding protein from intestinal mucosa has been isolated from guinea pig [173]. While similar in many respects to the serum transferrin of guinea pig (binds two Fe and has a molecular weight of ~80,000), it is different in its EPR and visible absorption spectra and amino acid composition and consists of two subunits. A transferrin has been isolated from rat intestinal mucosa; its iron binding properties are different from those of rat serum transferrin [173]. Additional studies of iron uptake by reticulocytes have appeared [174,175]. The ovotransferrin and serotransferrin of complexes with Fe(III), Cu(II), Co(III) and Mn(III) all show resonance enhanced bands in the Raman spectrum near 1600, 1500, 1270 and 1170 cm^{-1} [177]. Based in part on data from a copper model compound, tyrosine has been identified as a ligand. Mixed complexes of VO^{2+} and Fe^{3+}, and VO^{2+} and Cr^{3+} with serotransferrin have been examined [178]. Contrary to other reports (see section B iii), the data suggest that at physiological pH there is a preferential binding of Fe^{3+} to one site or conformation of the transferrin molecule. (N.D.C., October, 1976)

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