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The pH dependent properties of metallotransferrins: a comparative study

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The dependence on pH of the absorption and circular dichroic spectra of iron(III), cobalt(III) and copper(II) transferrins has been (re)investigated. *In the alkaline region*, the CD profiles of iron(III) and cobalt(III) transferrin are essentially pH independent up to pH 11; only for very high pH values (pH > 11) is breakdown of the cobalt(III) and iron(III) transferrin derivatives observed, without evidence of conformational rearrangements. By contrast, the CD profiles of copper transferrin show drastic changes in shape around pH 10; these spectral changes, which are fitted to a pK_a of ~10.4, are interpreted in terms of a substantial rearrangement of the local environment of the copper ions at high pH. Although the CD spectra of copper transferrin at alkaline pH strictly resemble those observed upon addition of modifier anions, the mechanism of site destabilization in the two cases is different; at variance with the case of modifier anions, our results suggest that the high pH form of copper transferrin still contains the synergistic anion. A ^{13}C NMR experiment has confirmed this view. *In the acidic region*, iron(III) and cobalt(III) transferrins are stable down to pH ~6. For lower pH values progressive metal detachment is observed without evidence of conformational changes; around pH 4.5 most bound metals are released. In the case of the less stable copper–transferrin, metal removal from the specific binding sites is already complete around pH 6.0; in concomitance with release from the primary sites, binding of copper ions to secondary sites is observed. Additional information has been gained from CD experiments in the far UV. The pH dependent properties of iron(III), cobalt(III) and copper(II) transferrin are discussed in the frame of the present knowledge of transferrin chemistry, particular emphasis being attributed to the comparison between tripositive and bipositive metal derivatives.

Keywords: circular dichroism, pH dependence, transferrin

Introduction

Transferrin is the key protein of iron metabolism in higher organisms, ensuring the transfer of the iron(III) ions, through biological fluids, from uptake to utilization sites (Harris & Aisen 1989, Crichton & Ward 1992). In addition, transferrin acts as a general ligand of several tripositive, tetrapositive and bipositive metal ions, some of these being of medical or toxicological concern (Messori & Kratz 1994). The crystal structure of tranferrins has eventually been solved to a good resolution by the groups of Baker and Lindley (Bailey *et al.* 1988, Anderson *et al.* 1989, Baker & Lindley 1992, Day *et al.* 1993, Lindley *et al.* 1993).

The transferrin protein comprises two structurally similar lobes each capable of binding, with high affinity, one iron(III) equivalent. The crystallographic results suggest that metal binding occurs through the closing of two protein domains around the metal ion by a hinge movement (Baker &

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Lindley 1992, Lindley *et al*. 1993); the closing process is promoted by the so called synergistic anion, which physiologically is (bi)carbonate. The environment of the bound iron(III) ions in the two sites is virtually identical, consisting of a distorted octahedron with one His, one Asp, two Tyr and the bidentate carbonate as ligands.

Remarkably, anions such as perchlorate, chloride, thiocyanate, etc. may act as modulators of the protein conformation in solution through binding to specific trigger sites on the protein surface (modifier anions) (Folajitar & Chasteen 1982, Williams *et al*. 1982, Thompson *et al*. 1986). The relevant effect of *modifier anions* on the protein structure highlights a crucial feature of transferrin chemistry: the importance of electrostatic interactions in maintaining the correct folding of the protein and in governing the opening/closing process. Similar effects are observed when considering the role of pH on the protein conformation; for instance, Bianconi *et al*., by contrast with the current opinion, have proposed that it is the pH and not the presence of the metal ion which is the true reason for the protein closing process (Congiu Castellano *et al*. 1994).

To shed light on these still controversial issues, we decided to re-investigate the pH dependent spectral properties of iron(III), copper(II), and cobalt(III) transferrins. We used CD spectroscopy because this technique represents an easy, direct and sensitive tool to follow possible conformational changes around the iron(III), cobalt(III) and copper(II) centers when monitoring the visible region (Woody 1995). Simultaneously, analysis of the CD spectrum in the UV region provides information on the occurrence of conformational changes in the overall protein scaffold (Woody 1995).

Apart from iron(III), which is the true physiological metal we have investigated the copper(II) and the cobalt(III) derivatives since the former represents a good example of a bipositive metal derivative, whereas the latter, owing to the known kinetic inertness of cobalt(III) complexes, might allow detection of partially unfolded protein states preceeding metal release.

Experimental procedures

Human serum apotransferrin was prepared in Sclavo (Siena) and further purified according to the reported procedure (Schlabach & Bates 1975). The lyophilized protein was dissolved in a buffer containing 20 mM NaH_2PO_4, 20 mM NaCl and 1 mM $NaHCO_3$, pH 7.4. Protein concentration was determined by measuring the intensity of the characteristic UV band at 280 nm ($\epsilon 280 = 91\,200$ M^{-1} cm^{-1}). Iron, cobalt, copper and indium chloride were all of analytical grade. The cobalt(III) derivative was prepared by addition of two equivalents of cobalt(II) chloride to an apotransferrin solution, followed by oxidation with a slight excess of hydrogen peroxide (Zweier *et al*. 1981). Formation of the adducts was checked by analysis of the characteristic absorption bands in the visible spectra. The pH was adjusted to the desired values by addition of small amounts of either sodium hydroxide or hydrochloric acid.

The UV visible spectra were recorded on a double beam CARY 3 instrument operating at room temperature. The CD spectra were run on a JASCO J500 spectropolarimeter. Samples for absorption and CD measurements were about 5×10^{-5} M in protein. When working in the 200–250 nm region the samples were further diluted to final protein concentration of ~1 μM and analyzed with 1 mm pathlength cuvettes under an intense nitrogen flow.

The ^{13}C NMR spectra were acquired on a Bruker MSL 200 instrument. Samples for ^{13}C NMR measurements were 1.5 mM in protein, 20 mM phosphate buffer, 20 mM NaCl, pH 7.4. The volume was about 0.5 ml with 10% D_2O. ^{13}C enriched sodium bicarbonate (3 mM) was added to the samples before addition of two equivalents of copper ions. Typical ^{13}C NMR acquisition parameters used in this study were as follows: 70° pulse length, 3.0 s repetition time, and a sweep width of 20 000 Hz. A line broadening of 5–10 Hz was applied to all ^{13}C data prior to processing.

Results

Iron(III) transferrin

The visible CD spectra of diferric transferrin (Fe_2Tf) at various pH values are shown in Figures 1A and 1B. At pH 7.4 the visible spectrum is characterized by an intense negative band at 445 nm plus a positive band at higher energy (around 330 nm); these bands have previously been assigned as LMCT transitions (Prados *et al*. 1975). The absorption spectra of the same samples are shown in Figures 2A and 2B.

The spectrum of Fe_2Tf is essentially pH insensitive over the range 11.5–6.5 apart from a slight intensity increase of the main LMCT transition between pH 7 and pH 9. Only for very high pH values (pH > 12) does almost complete disappearance of the characteristic CD transitions occur, suggesting that the bound iron(III) ions are released (Figure 1B); in any case no sign of conformational

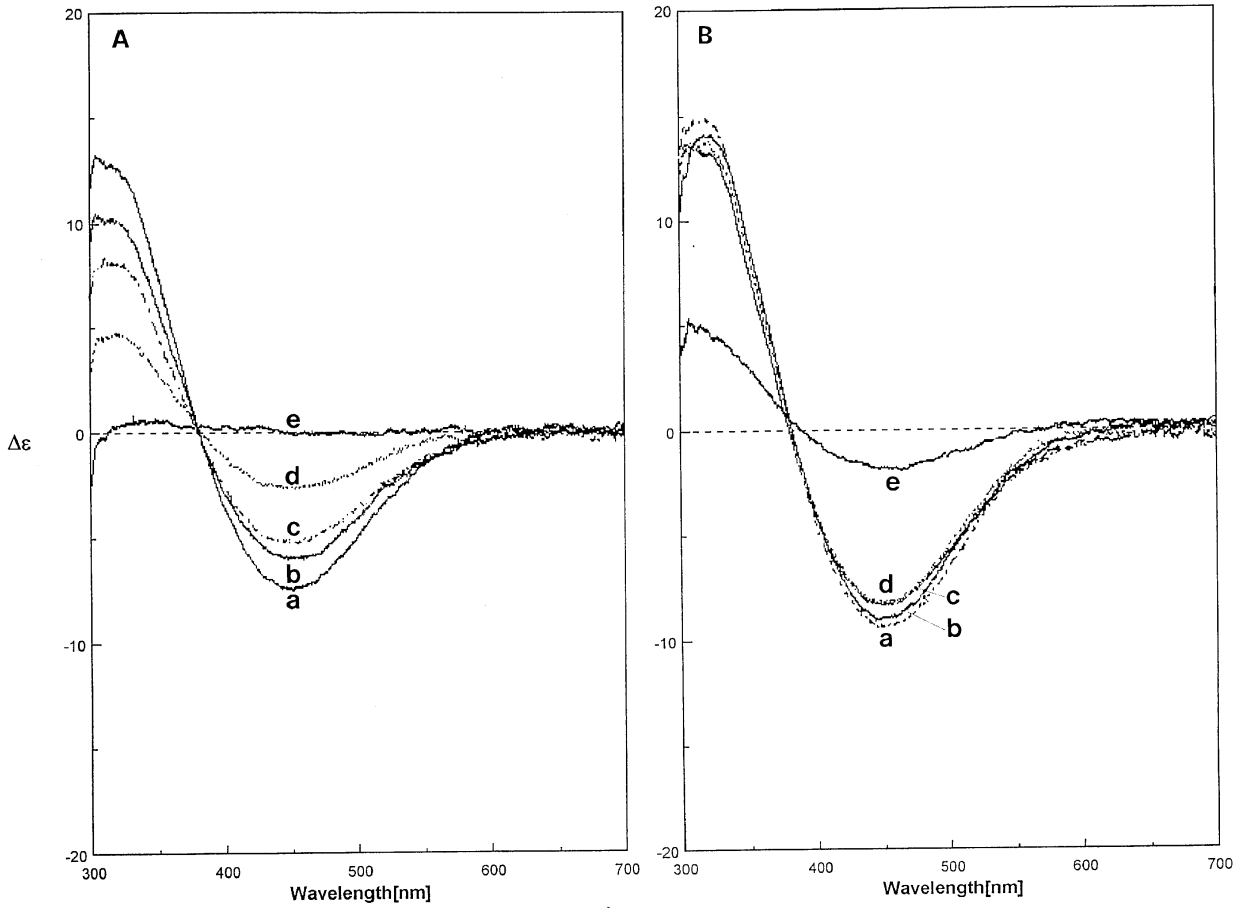

Figure 1. CD spectra of diferric transferrin in the acidic (A) and alkaline (B) regions. Figure 1A: (a) pH 7.4, (b) pH 6.5, (c) pH 6.0, (d) pH 5.5, (e) pH 4.5; Figure 1B: (a) pH 8.4, (b) pH 9.5, (c) pH 10.5, (d) pH 11.5, (e) pH 12.5. Conditions: starting buffer, 20 mM NaH_2PO_4, 20 mM NaCl and 1 mM $NaHCO_3$, pH 7.4; protein concentration 5×10^{-5} M. Spectra were usually recorded after approximately two hours equilibration.

changes of the chromophore is observed at high pH. The same picture emerges from the electronic absorption spectra (Figure 2B).

Conversely, lowering the pH below 6 causes, as expected, progressive detachment of the iron(III) ions from the protein. At pH 4.5 almost no residual CD or absorption band of the bound iron is observed (Figure 1A; Figure 2A). Again, the decrease in pH, does not affect the conformation around the bound metals. In other words no significant change in the spectral shape is detected with decreasing pH. except for the obvious intensity variations. A slight shift in the position of the high energy absorption band may be noticed, presumably arising from the fact that the spectral features of the two sites are slightly different and that the sites depopulate sequentially.

Cobalt(III) transferrin

The visible CD spectra of dicobalt(III) transferrin (Co_2Tf) at various pH values are shown in Figures 3A and 3B. The corresponding absorption spectra of the same samples are shown in Figures 4A and 4B. The CD spectrum of dicobalt(III) transferrin at pH 7.4 is characterized by three main transitions, respectively located at 370, 450 and 680 nm. The CD spectrum does not change over the pH range 7.0–11. At pH 11.5 a significant decrease in the intensity of all the CD transitions is observed without evidence of conformational variations. This behavior is ascribed to the progressive release of the bound cobalt(III) ions. The process is kinetically slow and takes several hours to reach completion. The spectrum of the same sample recorded the following day

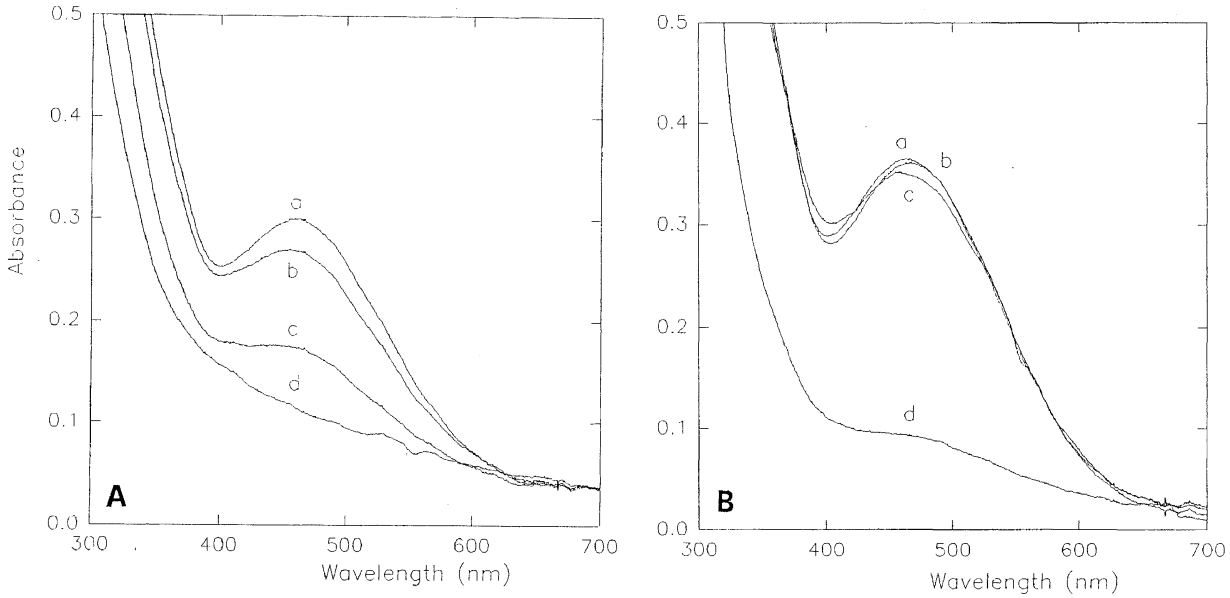

Figure 2. Electronic spectra of diferric transferrin in the acidic (A) and alkaline (B) regions. Figure 2A: (a) pH 6.5, (b) pH 6.0, (c) pH 5.5, (d) pH 4.5; Figure 2B: (a) pH 9.5, (b) pH 10.5, (c) pH 11.5, (d) pH 12.5. Conditions as in Figure 1.

shows a much larger decrease in the intensity of the transitions, suggesting that at least 30% of the bound cobalt(III) ions have been released (data not shown). The electronic spectra substantially reproduce the CD results.

The behavior of cobalt(III) transferrin in the acidic limb is similar to that of iron(III) transferrin, although the former is slightly more acid-sensitive. For pH < 7 a rather regular decrease in the intensity of the CD bands with decreasing pH is observed, without evidence of conformational changes. At pH 4.5 virtually all cobalt(III) ions have been released. Again, the electronic spectra confirm such a description (Figure 4).

Copper transferrin

Copper transferrin is characterized by a well known CD spectrum with three positive bands respectively located at 330 nm, 440 nm and 600 nm (Prados *et al.* 1975, Bertini *et al.* 1988). The two former bands have been assigned as ligand to metal charge transfer bands; the latter one corresponds to the d–d transition of the bound copper(II) ions (Bertini *et al.* 1988). The intensity of the d–d transition has been shown to depend critically in sign, intensity and shape on the presence of modifier anions (Bertini *et al.* 1988).

The visible CD spectra of copper transferrin are virtually pH independent between pH 7.4 and pH 9.5 (Figure 5B). For pH values lower than 7.4 progressive loss of the copper ions from the specific binding sites is observed (Figure 5A). Copper release is complete around pH 5.5. The detached copper ions apparently migrate to secondary sites, as witnessed by the appearance of a broad negative CD band at 590 nm. A similar picture emerges from the electronic spectra in the acidic limb (Figure 6A).

More intriguing is the behavior of copper transferrin in the high pH region. For pH values higher than 9.5 a series of spectral changes are observed indicated by the progressive disappearance of the band at 440 nm and the inversion of the 600 nm transition. The process is virtually complete around pH 11 and can be fitted to a single p*K*a of ~10.4. In parallel, the electronic spectra of copper transferrin at increasing pH show the progressive decrease of the charge transfer transition at 430 nm (Figure 6). Upon increasing the pH further the CD spectrum is virtually abolished.

Overall these results suggest that, by contrast with diferric and dicobalt transferrin, dicopper transferrin undergoes an important conformational change of the copper chromophore at high pH. It is probable that such a change involves loss, or drastic weakening, of the tyrosine coordination to the metal. Apparently the high pH conformation predominates between pH 10 and 11.5.

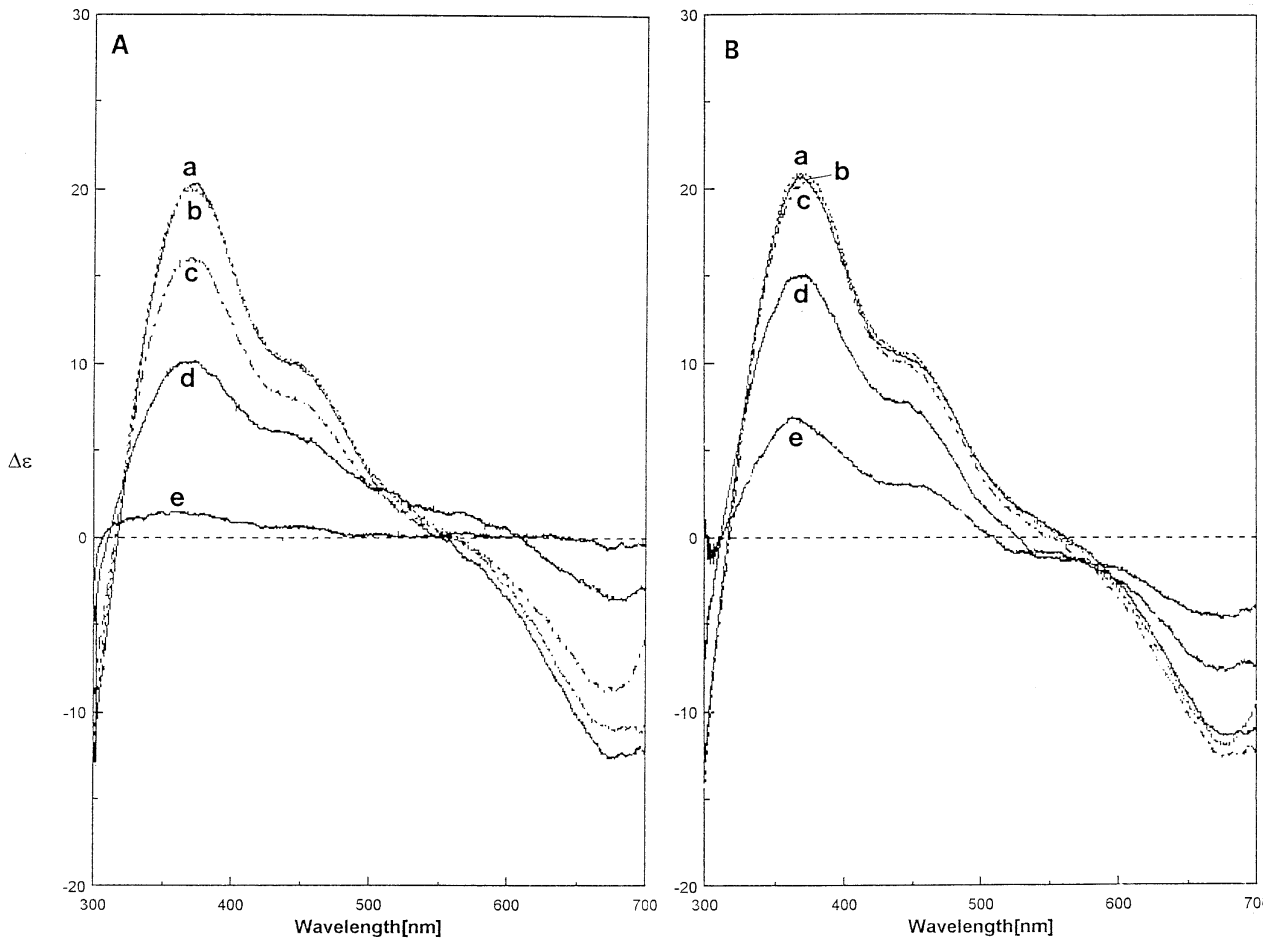

Figure 3. CD spectra of dicobalt transferrin in the acidic (A) and alkaline (B) regions. Figure 3A: (a) pH 8.5, (b) pH 7.5, (c) pH 6.5, (d) pH 5.5, (e) pH 4.5; Figure 3B: (a) pH 8.5, (b) pH 9.5, (c) pH 10.5, (d) pH 11.5, (e) pH 12.5. Conditions as in Figure 1.

Further experiments on copper transferrin

In order to highlight better the peculiar behavior of dicopper transferrin in the alkaline region we performed an additional series of experiments:

Addition of excess bicarbonate to copper transferrin at high pH. Addition of a tenfold excess bicarbonate to a solution containing comparable amounts of 'normal' dicopper transferrin and 'high pH' dicopper transferrin (pH = 10) did not affect the equilibrium between the two species as shown by CD spectroscopy; indeed, the intensity of the CD transition at 600 nm was not modified in any way upon bicarbonate addition. In our opinion this experiment means that the synergistic anion is part of both adducts. Were it not part of the high pH species, a drift of the equilibrium toward 'normal' dicopper transferrin would be observed upon increasing the concentration of bicarbonate by a factor of 10. No spectral change was observed even for bicarbonate concentrations as high as 60 mM. This result suggests that the high pH copper complex is still a ternary complex even if the tyrosinate–metal bonds are apparently broken. The same does not hold for dicopper transferrin at low pH; in this case addition of excess bicarbonate to copper transferrin, keeping constant the pH, results in a substantial recovery of the LMCT transitions at 430 and 600 nm.

Displacement of copper by excess indium(III) ions. In a second experiment we added a slight excess of indium(III) chloride to a copper transferrin sample at pH 10 and recorded the visible CD spectrum the

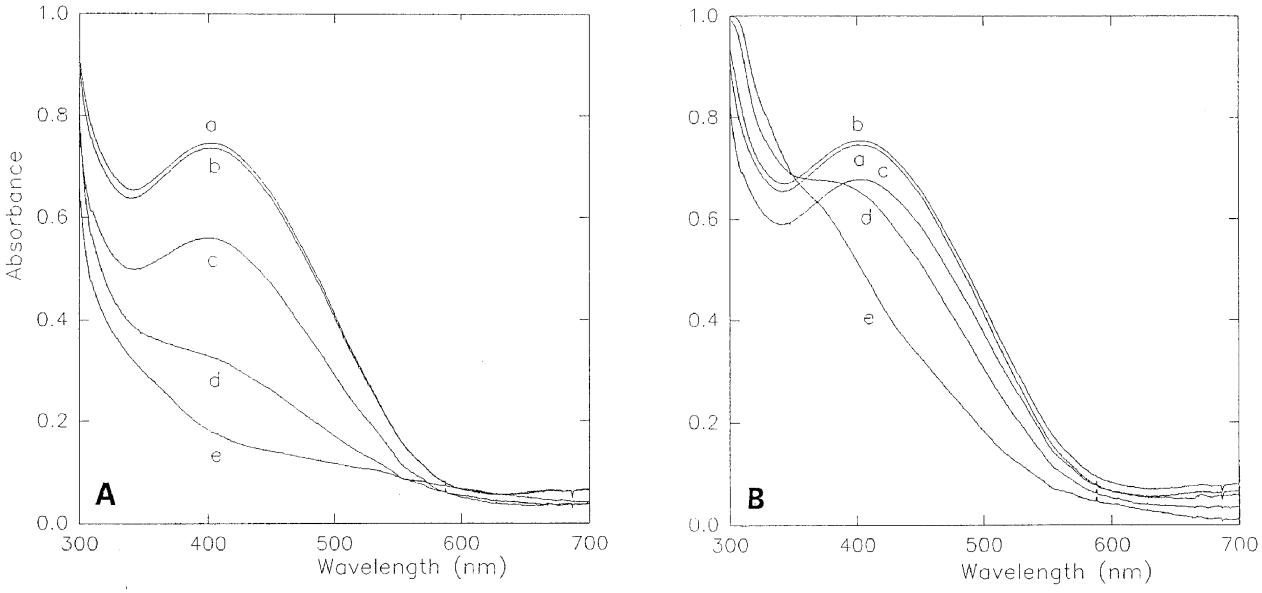

Figure 4. Electronic spectra of dicobalt transferrin in the acidic (A) and alkaline (B) regions. Figure 4A: (a) pH 8.5, (b) pH 7.5, (c) pH 6.5, (d) pH 5.5, (e) pH 4.5; Figure 4B: (a) pH 8.5, (b) pH 9.5, (c) pH 10.5, (d) pH 11.5, (e) pH 12.5. Conditions as in Figure 1.

following day. Indium(III) ions are known to bind transferrin tightly at the iron sites (Harris *et al.* 1994, Battistuzzi *et al.* 1995) and are therefore able slowly to displace the bound copper ions. The CD spectrum shows the disappearance of the residual tyrosinate band at 430 nm and a shift of the negative band at 590 nm toward higher energies (~540 nm, data not shown). The latter feature suggests that indium(III) ions do affect the spectral properties of the high pH copper species. The final spectrum is similar but not identical to a spectrum obtained upon addition of excess indium(III) chloride to a copper transferrin sample at pH 8. We interpret these results in terms of copper displacement (both 'normal' and 'high pH' copper) from the specific sites and subsequent binding to secondary sites.

^{13}C NMR experiments. In a third experiment we monitored the direct binding of the synergistic anion to the copper center through ^{13}C NMR spectroscopy. It is known from the literature that ^{13}C NMR spectroscopy can be profitably employed to monitor the resonance of the bound synergistic anion in diamagnetic metallotransferrins (Zweier *et al.* 1981, Bertini *et al.* 1986a, Aramini & Vogel 1993). If the metal is paramagnetic as in the case of iron(III) or copper(II) the ^{13}C NMR signal of bound (bi)carbonate is broadened beyond detection (Bertini & Luchinat 1986). Our experiment was conceived in such a way as to reveal the possible release at high pH of ^{13}C enriched (bi)carbonate from a dicopper transferrin preparation. Apotransferrin was mixed at pH 8 with a twofold stoichiometric amount of ^{13}C enriched bicarbonate, and the ^{13}C NMR spectrum was recorded. Natural abundance ^{13}C signals from the protein and the signal of free bicarbonate were easily detected in the carbonyl region (Figure 7a). Two equivalents of copper ions were then added to the sample and formation of dicopper transferrin was checked through absorption spectroscopy. As expected, formation of dicopper transferrin results in disappearance of the ^{13}C NMR signal of free bicarbonate (Figure 7b). Subsequently, the pH was raised to 10.0 and the ^{13}C NMR spectrum was recorded once more (Figure 7c). Again, no signal of free (bi)carbonate is observed, suggesting that the anion is still bound to copper.

CD spectra in the UV: ApoTf, Cu_2Tf; Fe_2Tf, Co_2Tf

To complete our description of the pH dependent spectroscopic properties of metallotransferrins we performed a series of CD spectra in the far UV region. CD spectroscopy in the far UV region is indeed a valuable tool for detecting changes in the secondary structure of proteins (Woody 1995). The far UV CD spectra of apotransferrin and of the

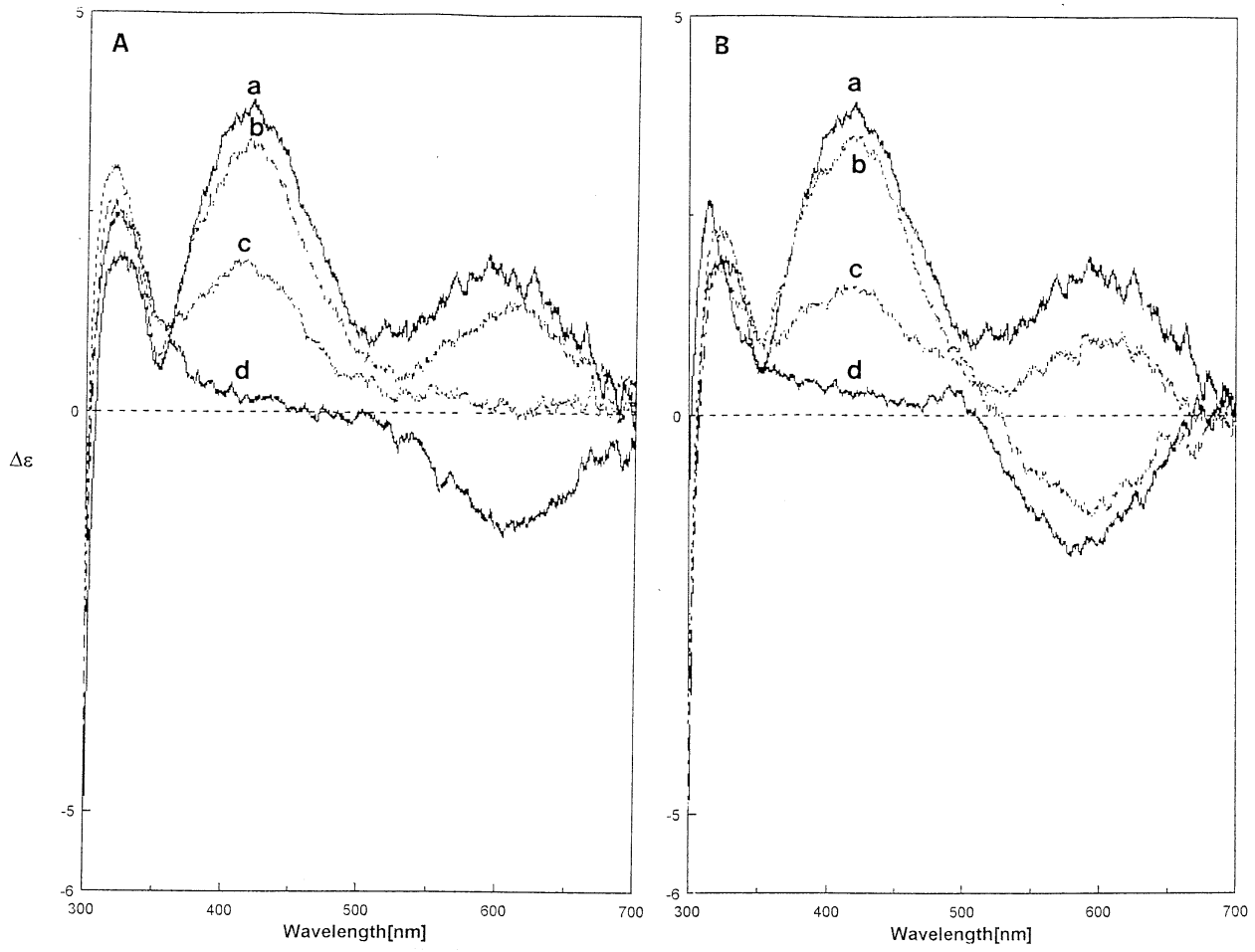

Figure 5. CD spectra of dicopper transferrin in the acidic (A) and alkaline (B) regions. Figure 5A: (a) pH 8.5, (b) pH 7.4, (c) pH 6.5, d) pH 5.5; Figure 5B: (a) pH 8.5, (b) pH 9.5, (c) pH 10.5, (d) pH 11.5. Conditions as in Figure 1.

three metallotransferrins are very similar to each other between pH 5 and pH 9 indicating that no significant secondary structure change occurs upon metal binding; this is in agreement with previous observations (Nagy & Lehrer 1972, Mazurier *et al.* 1976). Raising the pH up to 11 causes an intensity increase and a shift of the negative band at 210 nm. For pH values > 12 the typical spectrum of a random coil protein appears. In Figure 8 we show as an example the far UV CD spectra of diferric transferrin at different pH values.

Discussion

The role of pH on transferrin

There is considerable interest in understanding the pH dependent properties of transferrin, primarily because exposure of the protein to a low pH environment is believed to represent the actual mechanism of metal destabilization and release *in vivo*. Only around physiological pH does a correct conformation of the protein in solution exist that favors metal binding; this native conformation is stabilized by a network of electrostatic and hydrogen bond interactions, as it can be deduced from the X-ray description of the metal binding sites (Day *et al.* 1993). As we move far away from physiological pH progressive perturbation of the native conformation of the holoprotein takes place. Such perturbation is believed to bring about, first, disruption of the metal environment, then disruption of the overall tertiary and secondary structure. These two steps are usually observed sequentially both in the acidic and alkaline region for all metallotransferrins.

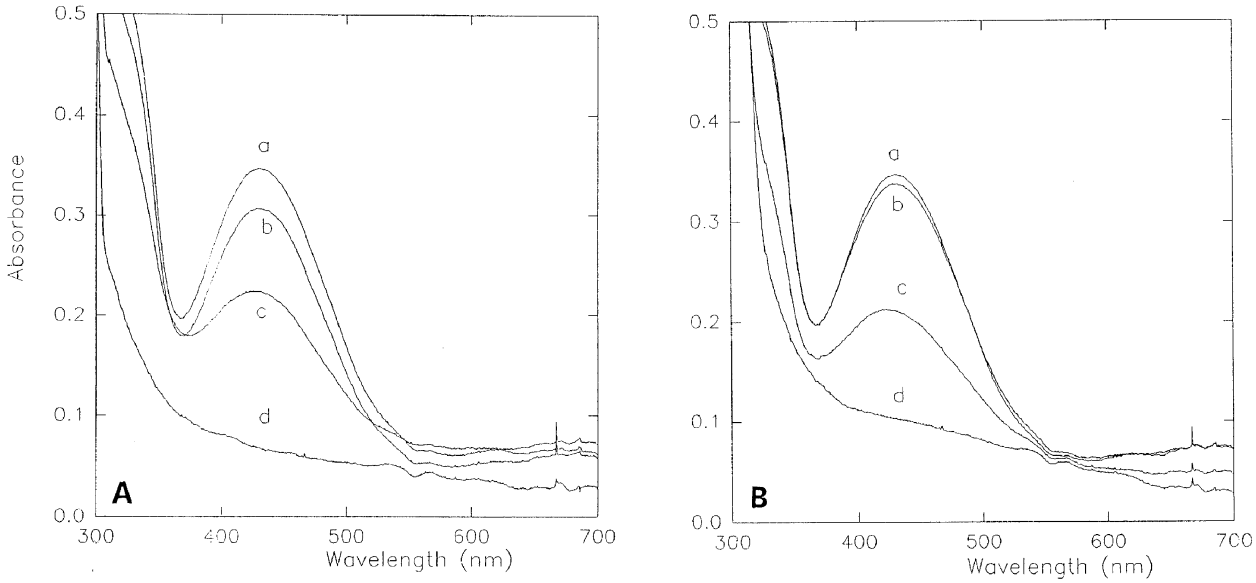

Figure 6. Electronic spectra of dicopper transferrin in the acidic (A) and alkaline (B) regions. Figure 6A: (a) pH 8.5, (b) pH 7.4, (c) pH 6.5, (d) pH 5.5; Figure 6B: (a) pH 8.5, (b) pH 9.5, (c) pH 10.5, (d) pH 11.5. Conditions as in Figure 1.

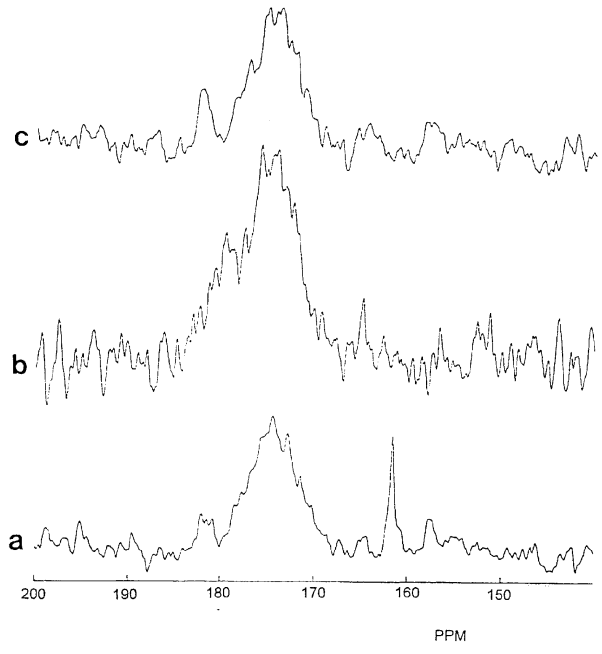

Figure 7. ^{13}C NMR spectra of: (a) apotransferrin at pH 8.0; (b) dicopper transferrin at pH 8.0; and (c) dicopper transferrin at pH 10.0. Conditions: 1.5 mM protein concentration, 20 mM phosphate buffer, 20 mM NaCl. The volume was about 0.5 ml with 10% D_2O and 3 mM ^{13}C enriched sodium bicarbonate.

More intriguing is the interpretation of these processes in terms of molecular events. By lowering the pH crucial basic groups within the metal binding cavity are protonated, potential candidates being Asp and Glu carboxylate groups, the synergistic anion and the metal bound phenolates. Progressive protonation results in weakening of the native conformation, opening of the site, loss of the synergistic anion, breaking of the metal–tyrosine bonds, and eventual release of the metal. Unfortunately all these events occur concomitantly without apparent formation of intermediates so that the exact mechanism of release cannot be followed in detail. Conversely, upon moving towards the *alkaline region*, progressive deprotonation of the acidic groups hanging in the cavity occurs. Main candidates for deprotonation are the positively charged groups such as histidines, lysines and arginines and the non-coordinated tyrosine residues. In a way, the effect of high pH should parallel that of modifier anions since both processes determine charge neutralization. Remarkably, Chasteen and coworkers have shown that the effect of modifier anions on transferrin is mimicked by chemical modification of the lysine groups within the cavity (Thompson *et al.* 1986).

Our study has stressed this 'open' issue of transferrin chemistry with the help of new spectroscopic data. In particular the following results have emerged:

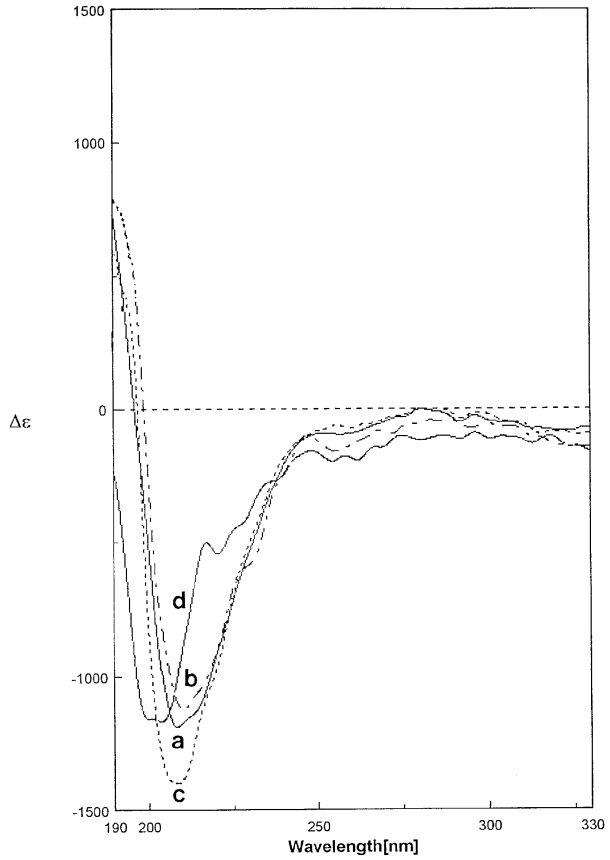

Figure 8. Far UV CD spectra of diferric transferrin at the following pH values: (a) pH 5; (b) pH 8; (c) pH 11; (d) pH 12.5. Conditions: 1 μM protein concentration; other conditions as in Figure 1.

(i) iron(III) and cobalt(III) transferrin show a rather parallel behavior, both being stable between pH 6.5 and pH 11.
(ii) copper transferrin is less acid stable and starts breaking down around pH 6.5.
(iii) copper transferrin undergoes an important conformational change in the alkaline region with a pKa of ~10.4.
(iv) the high pH copper transferrin species, having apparently lost phenolate coordination, still contains the synergistic anion.
(v) no relevant change of the secondary structure is observed over the pH range 4.5–10

Noticeably, iron(III) and cobalt(III) transferrins are stable at alkaline pH values. Their spectral features remain virtually unmodified up to pH 11, apart from a slight intensity increase of the LMCT transition of iron transferrin between pH 7 and pH 9. For very high pH values disruption of the metal site occurs followed soon after by collapse of the secondary structure. In the acidic region iron(III) transferrin and cobalt(III) transferrin are stable down to pH 6 and 6.5 respectively, then start releasing the bound metals without evidence of conformational changes. No significant change of the secondary structure is detected over the pH range 10–4. All these results are in good agreement with the current view of the pH dependent properties of transferrins. At variance, copper(II) transferrin is more labile both in acidic and alkaline pH, in agreement with the lower stability of the ternary complex. Interestingly, at high pH, a new copper species has been described, that implies a drastic rearrangement of the copper chromophore without loss of the synergistic anion (see below).

The peculiarity of copper transferrin

Despite the fact that copper transferrin is of limited physiological significance, this derivative represents a valuable probe for the metal binding sites of transferrin. Thus, the copper derivatives of transferrin have been intensely investigated by a variety of techniques such as EPR (Aasa & Aisen 1968, Zweier & Aisen 1977), ESEEM (Zweier *et al.* 1979, Eaton *et al.* 1989), ENDOR (Roberts *et al.* 1983), NMRD (Bertini *et al.* 1985), and EXAFS (Mangani & Messori 1992). The X-ray structure of copper lactoferrin has been reported to a high resolution (Smith *et al.* 1992).

We have previously reported a CD study of copper transferrin and demonstrated the sensitivity of the metal sites to 'chaotropic' anions such as perchlorate, thiocyanate and chloride (Bertini *et al.* 1988). Here, the copper derivative has been used to monitor the sensitivity of the metal sites to pH variations.

As noted above, the behavior of copper transferrin in the alkaline region is peculiar. A noteworthy observation is that the 430 nm LMCT transition, characteristic of phenolates, disappears with a pK_a of 10.4 suggesting that phenolate coordination to the metal is abolished or drastically weakened. On the other hand, the ^{13}C NMR spectra and some additional CD experiments indicate that the synergistic anion remains bound to the copper ion. It is inferred that at high pH the copper ions are still bound to His, to the synergistic anion and possibly to Asp. Such a high pH copper complex should correspond to the colorless high pH copper species previously described by EPR (Aasa & Aisen 1968).

At a first glance, the effect of high pH paral-lels that of modifier anions since in both

cases copper transferrin responds to external perturbations with characteristic, and similar, CD spectral changes. We have demonstrated, however, that even if the spectral changes in the two cases are nearly the same, the underlying mechanism of site perturbation is different. Indeed, in the case of modifier anions, site perturbation results in destabilization of the specific sites and eventual copper migration to secondary sites (Bertini *et al.* 1988); by contrast, at alkaline pH, the copper site is heavily perturbed but not destroyed. Only upon addition of metals that bind transferrin more tightly—indium(III) ions in our case—do copper ions migrate to secondary sites.

A unified picture for the pH dependent properties of metallotransferrins

It is generally accepted that native diferric transferrin is highly stable and does not exhibit significant spectral changes over a wide pH range (namely from 6 to 11). Loss of iron in the alkaline region, occurring for pH >12, is accompanied soon after by loss of the secondary structure; on the other hand, low pH metal release is probably modulated by protonation of the synergistic anion and/or some nearby residue.

Even in the absence of systematic studies of the pH dependent properties of metallosubstituted transferrins, several indications exist in the literature of the comparative stability of the various metal–transferrin adducts with pH. Generally, there is a good correlation between the stability constant of the metal–protein complex under physiological conditions and its pH range of existence; the higher the affinity of the metal, the larger the pH stability of the resulting adduct. Thus, tight metal(III) transferrin complexes such as those with gallium(III) and indium(III), exhibit a pH stability range similar to that of iron(III) transferrin. By contrast, the weaker metal(II) derivatives are less stable both at acidic and basic pH. It is known that in the acidic region metal(II) derivatives such as cobalt(II) (Bertini *et al.* 1986b), copper(II) (Zweier & Aisen 1977), oxovanadium(IV) (Chasteen *et al.* 1977) and cadmium(II) (Sola 1990) transferrin break down around pH 7. The same complexes manifest their lower stability in alkaline pH as conformational heterogeneity. For instance, cobalt(II), copper(II) and oxovanadium(IV) transferrin have been shown to produce a number of spectroscopically detectable conformers at high pH. A p*K*a around 10 is usually detected for the high pH conformational equilibria, a value which is probably correlated to deprotonation of a strategic lysine residue. In all cases the conformational transition of the metal site precedes metal release.

A borderline behavior is shown by the transferrin complexes of large tripositive and tetrapositive cations such as thallium, thorium or lanthanides. These cations, in fact, give rise to complexes of intermediate stability and are less resistant to pH variations than are the Fe(III), Ga(III), In(III) or Al(III) complexes. Particularly significant is the case of Tl(III) transferrin, which breaks down around pH 10 (Bertini *et al.* 1983); conversely thorium transferrin has been shown to exhibit an important spectral p*K*a of ~9.5 (Harris *et al.* 1981). The derivatives with lanthanides are usually stable between pH 7.5 and 10.5 (Messori *et al.* 1986, Messori & Piccioli 1991, O'Hara & Koenig 1986).

The present study on iron(III), cobalt(III) and copper(II) transferrin reinforces the above picture. The deduced pattern of relative stabilities of iron, cobalt and copper transferrin is shown in Figure 9. The graph reports the normalized intensity of the main LMCT transition of the three metallotransferrins as a function of pH. It is apparent that cobalt(III) transferrin behaves very similarly to iron transferrin (although it is slightly less stable at acidic pH) whereas copper transferrin is a typical example of a bipositive metal complex. Particularly significant is the difference between the copper(II) and the cobalt(III) derivatives in the alkaline region,

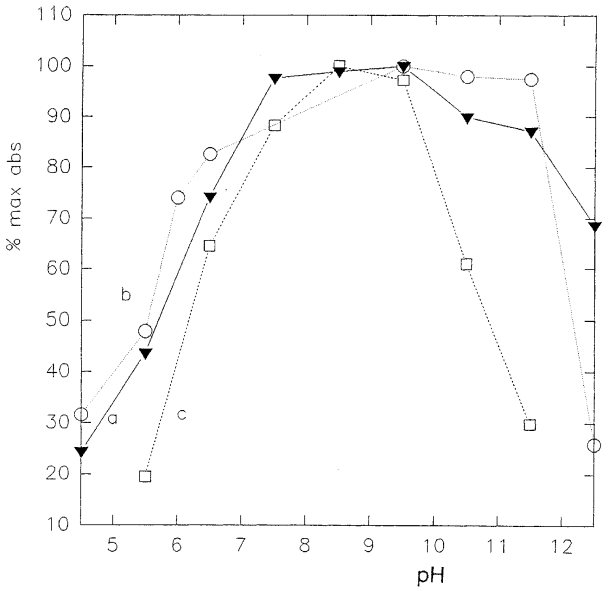

Figure 9. Dependence on pH of the intensity of the main electronic absorption band in three different metallotransferrins: (a) dicobalt transferrin; (b) diferric transferrin; (c) dicopper transferrin. Values are normalized to the maximum value for each derivative.

where copper transferrin gives rise to a high pH conformer with distinctive spectral features.

All the above considerations suggest the existence of a crucial residue in close proximity to the metal site, with a pK_a around 10.4, yet the exact identity of this group has not been ascertained. It is probably a nearby Lys or Arg residue; deprotonation of this group is believed to alter considerably the local stability of the metal site. If the metal–protein ternary complex is sufficiently tight, no significant effect on the spectral properties of the bound metal is detected; conversely, if the metal–protein interaction is not as strong, destabilization and rearrangement of the site occurs, according to the binding preferences of the metal. Alternatively, site destabilization may result in hydrolysis and loss of the metal as happens for thallium(III) transferrin.

Concluding remarks

The reinvestigation of the pH dependent properties of iron(III), cobalt(III) and copper(II) transferrin has provided further insight into the acid–base properties of this fascinating protein. We have shown that tight metal(III) derivatives are virtually pH independent between pH 6 and pH 11 whereas the weaker bipositive metal complexes are less stable both at acidic and alkaline pH. Remarkably, occurrence of a large conformational rearrangement at high pH, without loss of the synergistic anion, has been demonstrated for the copper derivative.

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