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UV Raman evidence of a tyrosine in apo-human serum transferrin with a low pK_a that is elevated upon binding of sulphate

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Abstract The binding of sulphate to human serum apo-transferrin has been examined by ultraviolet absorption and ultraviolet resonance Raman difference spectroscopies between pH 6.0 and 9.0. The ultraviolet absorption data reveals a negative feature at 245 nm that increases in magnitude with pH, with an apparent pK_a of 7.57, which the Raman difference data reveals to be due to tyrosine. The pK_a of this tyrosine is unusually low and is measured at 7.84 by the Raman difference method and is elevated to greater than 9.0 upon addition of sulphate. Previous studies on the N-lobe imply that Tyr 188 is the tyrosine with a low pK_a and also that Arg 124 is the primary binding site for the sulphate. The functional relevance may be that with sulphate bound, both carbonate binding and the deprotonation of Tyr will be disfavoured, and as a result so is iron binding.

Key words: Transferrin; Anion binding; Ultraviolet resonance Raman spectroscopy; Tyrosinate

1. Introduction

Transferrins are a group of iron binding proteins that bind iron only in the presence of synergistic anions (biologically, carbonate) [1]. They fold into two distinct homologous N- and C-lobes, each of which bind one Fe^{3+} ion and one synergistic anion. Each lobe is structurally further split, creating an inter-domain cleft, that closes upon iron binding. In human serum transferrin with iron loaded in both the N- and C-lobe, two Tyr residues, one Asp and one His residue, complex the Fe^{3+} ions in the lobes, with bidentate carbonate occupying the other coordination sites [2]. The binding of Fe^{3+} is also accompanied by a large structural change in each of the N- and C-lobes, which can adopt an open conformation in the apo form and a closed conformation when iron is bound [3–6]. The capture and release of Fe^{3+} are mediated by the binding of anions. Kinetic evidence suggests that the synergistic anion binds first, preparing the binding cleft for the Fe^{3+} ion [1]. Metal release is also affected by anion binding, which is thought to promote a structural change in the closed holo form [7].

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Abbreviations: hTf, human serum transferrin; apohTf, apo-human serum transferrin; hTf/2N, recombinant N-lobe human serum transferrin

The binding of both synergistic and non-synergistic anions to apo-human serum transferrin (apohTf) has been studied by ultraviolet (UV) difference spectroscopy and reveals a negative peak at 245 nm [8–11]. Analysis of the dependency of the magnitude of this negative feature with concentration of anion yields binding constants for a range of both divalent and monovalent anions. The non-synergistic anions are known to compete for the binding site with synergistic anions and interfere with Fe^{3+} uptake. The origin of the negative feature at 245 nm has been thought to be due to changes in the hydrogen bonding to a tyrosine residue near the iron binding site [12].

We report the application of ultraviolet resonance Raman (UVRR) spectroscopy to examine the sulphate binding to apohTf. UVRR spectroscopy gives specific Raman enhancement from tryptophan and tyrosine residues and the use of 244 nm radiation excitation is particularly well suited for the detection of tyrosinate, deprotonated tyrosine, as this species has a close lying electronic absorption and exhibits strong UVRR scattering [13]. We show that the appearance of negative UV absorption peaks upon the addition of anions at 245 nm is due to the protonation of a tyrosine in apohTf with an unusually low pK_a, and this pK_a is elevated upon binding of sulphate. Previous work on binding site and second shell tyrosine residue mutants of recombinant N-lobe human serum transferrin (hTf/2N) is analysed to explain the stabilisation for this low pK_a tyrosine.

2. Material and methods

Potassium sulphate, apohTf, HEPES and MES were purchased from Sigma. No attempt was made to further purify the apohTf. Buffers in the pH range of 6–9 were prepared by titrating a stock solution of 0.1 M HEPES and 0.1 M MES with sodium hydroxide. The concentration of the buffer was halved for accumulation of UV absorption difference spectra.

2.1. UV absorption difference spectra

UV absorption spectra were obtained on a Jasco V-560 UV/Vis Spectrophotometer. Approximately 12 μM solutions of apohTf were prepared by adding 50 μl of a 215 μM stock solution of apohTf in water to 800 μl of buffer. These solutions of apohTf were placed in both the sample and reference cuvettes and a baseline recorded between 300 and 235 nm. A 100 μl aliquot of a saturated solution of potassium sulphate (approximately 0.091 M) was then added to the sample cuvette and a 100 μl aliquot of buffer was added to the reference cuvette. Difference spectra were thus directly obtained by scanning from 300 to 235 nm.

2.2. UVRR spectra

Raman spectra were obtained using 2 mW of 244 nm radiation from an intracavity frequency-doubled argon ion laser (Coherent

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Innova 300 FReD). A Renishaw micro-Raman System 1000 spectrometer modified for use at 244 nm was used to acquire the spectra [14]. To minimise photodegradation, protein samples were circulated through a capillary tube, (inner diameter = 0.2 mm) by a mini-peristaltic pump (tubing inner diameter = 0.5 mm) from a reservoir. Spectra were accumulated over 8 min, (30×16 s integration) and analysed and subtracted using GRAMS 32 (Galactic Industries Corporation). Approximately 133 μM solutions of apohTf were prepared by adding 100 μl of a 400 μM stock solution of apohTf in water, to 200 μl buffer at pH values between 6 and 9. A UVRR spectrum was obtained and then 50 μl of a saturated solution of potassium sulphate was added to the sample reservoir. A UVRR spectrum of protein plus sulphate complex was obtained after a couple of minutes to allow the protein to mix with the sulphate.

3. Results

Fig. 1 shows UV absorption difference spectra of apohTf from before and after the addition of sulphate, between pH 6.0 and 9.0. The pH dependence of the 245 nm difference feature for whole apohTf is shown in Fig. 2 and reveals an apparent pK_a of 7.57. The possibility that the difference feature at 245 nm was due to tyrosine was further investigated by UVRR.

Fig. 3 shows the UVRR spectra of apohTf at pH 6.25 and pH 8.75. The spectra are labelled according to previous assignments [15,16]. The spectrum at pH 6.25 is dominated by tyrosine contributions, due to the 26 tyrosine residues in hTf, with the seven tryptophan residues making a moderate contribution. At pH 8.75 there is a noticeable increase in the tyrosinate feature at 1600 cm^{-1} (Fig. 3). The difference spectrum shown in Fig. 3, between the spectra at pH 8.75 and pH 6.25, shows only the features due to tyrosinate at 1601, 1560, 1208, 1170, and 853 and 831 cm^{-1}, from the Y8a, Y8b, Y7a, Y9a and the Y1 Fermi doublet tyrosinate modes, respectively. The minor features on the difference spectrum in Fig. 3 are due to changes in the buffer species between pH 8.75 and 6.25.

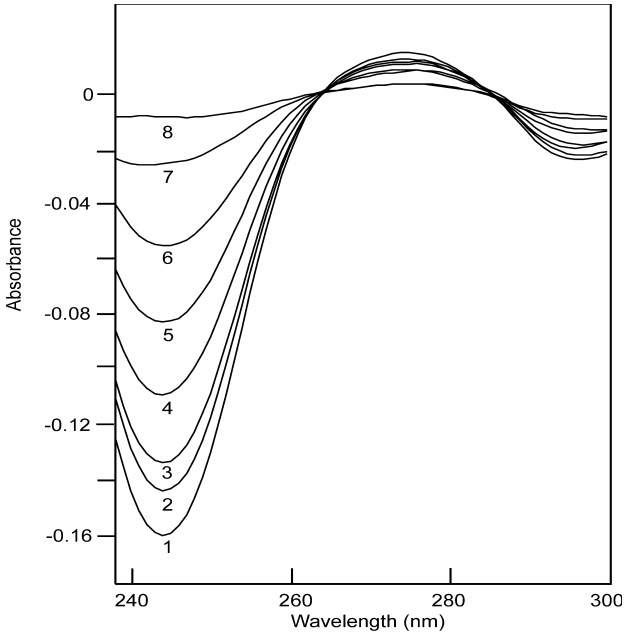

Fig. 1. UV absorption difference spectra produced by adding 100 μl 91 mM sulphate to 850 μl of approximately 12 μM apohTf at pH values (1) 8.75, (2) 8.26, (3) 8.07, (4) 7.75, (5) 7.53, (6) 7.25, (7) 6.77, (8) 6.25.

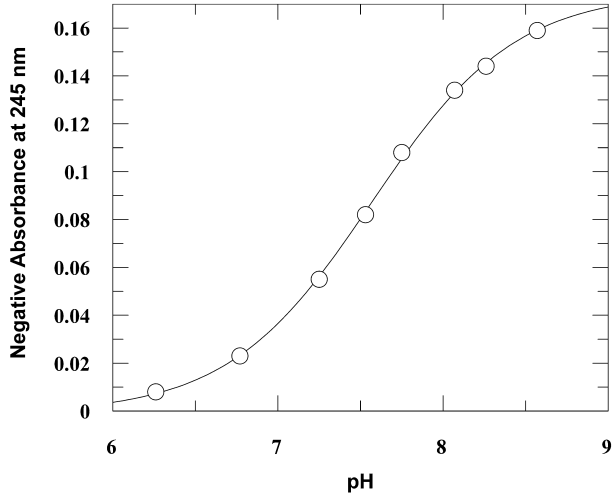

Fig. 2. pH titration curve of magnitude of negative 245 nm absorption difference feature, with pK_a fit at 7.57.

The effect on the UVRR spectrum of apohTf at pH 8.75 with the addition of sulphate anions is shown in Fig. 4 where the intense tyrosinate bands are observed to significantly reduce in intensity upon addition of sulphate. The difference spectrum in Fig. 4 shows negative tyrosinate features, with a positive sulphate feature at 980 cm^{-1}. The pH dependency of the magnitude of the large negative tyrosinate UVRR band at 1601 cm^{-1} is shown in Fig. 5 and reveals a pK_a of 7.84. This is the first conclusive evidence that the addition of sulphate ions to apohTf causes an elevation in the pK_a of a tyrosine residue.

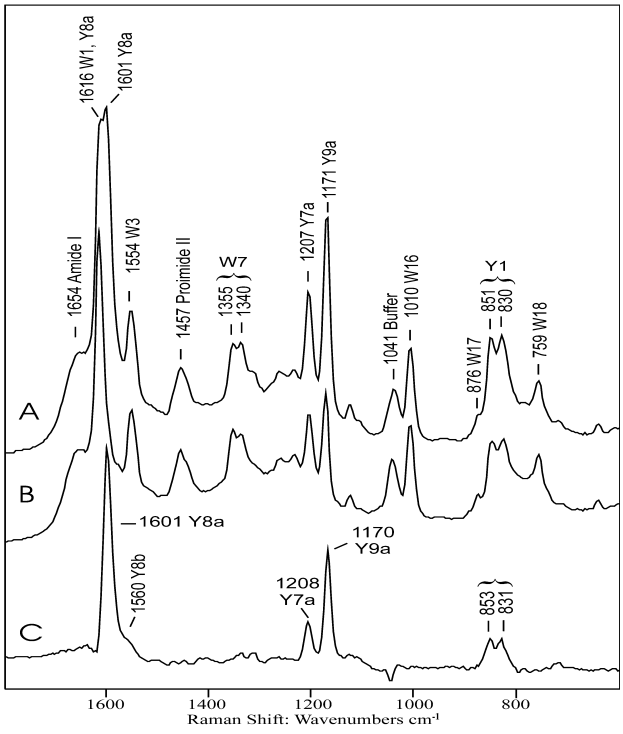

Fig. 3. 244 nm UVRR spectra of apohTf at (A) pH 6.25 and (B) 8.75. C: Difference spectrum of A minus B showing tyrosinate UVRR bands only, with minor features due to changes in buffer species.

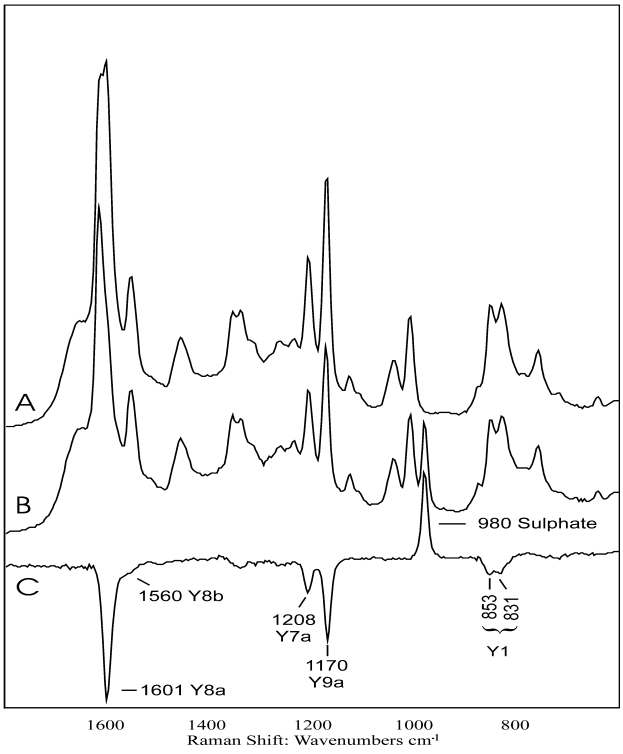

Fig. 4. 244 nm UVRR spectra of apohTf at pH 8.75 before (A) and after (B) addition of sulphate. C: Difference spectrum of A minus B showing negative tyrosinate bands and positive sulphate band.

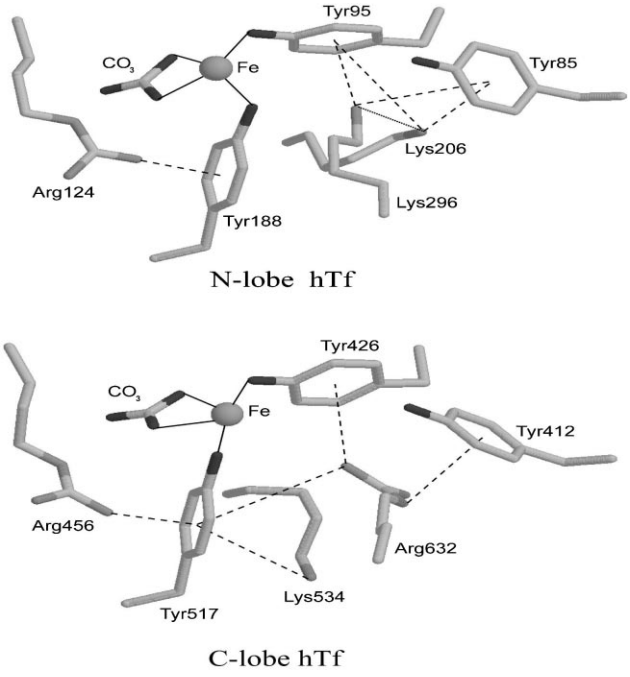

Fig. 6. Selected iron binding and second shell residues in the N- and C-lobe of hTf. Dotted line in N-lobe indicates the hydrogen bond between the dilysine trigger residues, Lys 206 and Lys 296. The dashed lines indicate possible cation-π interactions. Diagram produced in Rasmol. N-lobe structure from 1B3E and C-lobe from Harmon Zuccola [2]: resolution is 3.3 Å, *r* factor = 21.2%.

4. Discussion

A previous UV absorption study of the pH dependence of sulphate binding to apohTf/2N found a pK_a of 7.4 for the negative peak at 245 nm, which we assign to the reprotonation of a tyrosine residue [12]. The magnitude of difference in absorptivity at 246 nm after addition of sulphate, $-\Delta\varepsilon_{246}$, for apohTf/2N at pH 8.02, (-7559 M^{-1} cm^{-1}) [12], approaches that for the absorption at 241 nm for *N*-acetyl-L-tyrosinamide at high pH (11600 M^{-1} cm^{-1}) [13], giving more support for this assignment. A previous fluorescence study found a proton pK_a of 8.17 for whole apohTf, from a decrease in emission at ~230 nm with pH [17]. This feature also decreased with identical pH dependency upon the addition of hydrogen carbonate. The present UVRR study suggests that this fluorescence decrease is also due to tyrosinate, which is known to fluoresce at ~230 nm. Fluorescence emission spectra from tryptophan-containing proteins are usually dominated by tryptophan contributions, and caution has been advised in assigning fluorescent tyrosinate species at neutral pH [18]. The difference in pK_a measured by the UVRR method from the UV absorption, and especially the fluorescence method, may be explained by the nature of the different techniques. UV absorption and fluorescence probe differ between the aromatic residue ground state and excited states. The excited state can undergo energy transfer, complicating fluorescence measurements. Both these excited state methods can also have contributions from environmental effects that complicate it use in measurement of a single molecular species. This is in contrast with the UVRR method that only probes the aromatic ground state and gives detailed molecular information allowing for a direct measure of the relative populations of tyrosine and tyrosinate [13].

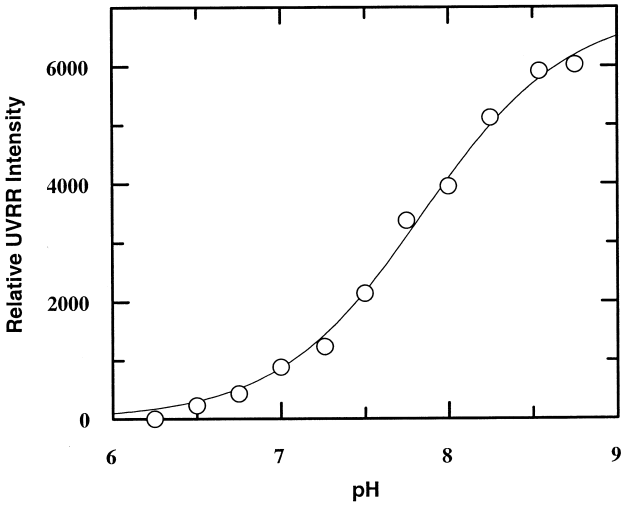

Fig. 5. pH titration curve of the relative UVRR intensity of the 1601 cm^{-1} difference feature, with pK_a fit at 7.84

The identification of tyrosinate as the electronic reporter event of anion binding still begs the questions, which tyrosine has the low pK_a and what are the structural factors that lead to its stabilisation? Studies on hTf/2N mutants provide clues to these questions. He et al. studied the sulphate binding of four tyrosine mutants, Y85F, Y95F, Y96F and Y188F of the hTf/2N [19]. Tyr 95 and Tyr 188 are iron binding residues and Tyr 85 is an important second shell residue (Fig. 6). All four

mutant proteins gave negative UV absorption difference features at 245 nm upon addition of sulphate. The Y188F mutant, however, displayed a significant decrease in both the maximum absorptivity, $\Delta\varepsilon_{max}$ and binding constant, K, which the authors took as implying that Tyr 188 is the principle electronic reporter of anion binding [19]. The present UVRR study would then suggests that Tyr 188 is the tyrosine residue with the unusually low pK_a in the N-lobe. The corresponding residue in the C-lobe of hTf is Tyr 517 (Fig. 6). The fact that a negative UV absorption feature is still present when Tyr 188 of hTf/2N is mutated to phenylalanine suggests possible further contributions from another tyrosine residue, most likely the close-lying Tyr 95 residue.

He et al. also examined the K206E, K296E, K206Q and K296Q mutants of hTf/2N and found that only the K206Q mutant displayed a negative 245 nm feature upon addition of sulphate [19]. Lys 206 and Lys 296 interact in the closed conformation through a strong hydrogen bond, with one or both lysine residues having a low pK_a (Fig. 6). At low pH, protonation of these two lysine residues is proposed to cause charge repulsion and trigger the opening of the binding pocket resulting in iron release. Thus, these residues are known as the dilysine trigger [20]. The lack of a 245 nm spectral signal in the dilysine trigger mutants upon addition of sulphate suggests a vital role for these residues in anion binding. In the crystal structure of the apohTf/2N, the interdomain cleft is in an open conformation and Lys 296 is remote from Tyr 188 [21], prompting He et al. to suggest that only in the closed conformation do Lys 296 and Tyr 188 interact [19]. He et al. postulated that the Lys 296 is a primary anion binding residue and Tyr 188 a major electronic reporting residue [19].

The closed conformations of the C- and N-lobes of hTf are, however, highly unlikely to exist as noticeable populations in solution. Small-angle X-ray scattering of apo-transferrins in solution, including the isolated N- and C-lobes of ovotransferrin, reveal that the lobes are in the open conformation [22]. It is therefore unlikely that Lys 296 makes a direct connection to Tyr 188 in apohTf/2N.

In the crystal structure of apohTf/2N, the protein is in the open conformation and the phenolic oxygen of Tyr 188 makes a hydrogen bond to the side chain amine group of Lys 206, which also makes a cation-π interaction with Tyr 95 [21]. It is tempting to speculate that Lys 206 also has a low pK_a and accepts the proton from the low pK_a Tyr 188 and can thus maintain the cation-π interaction with Tyr 95. Lys 206 belongs to the dilysine trigger, where one or both residues are thought to have a low pK_a [20].

The crystal structure of apo-ovotransferrin N-lobe complexed with sulphate ions has recently been solved and three sulphate ions are found in the interdomain cleft, which is in the open conformation [23]. In site 1, sulphate is bound to His 250, in site 2 the anion is bound to Arg 121 and in site 3 to the backbone amide nitrogen of Tyr 191. His 250 and Tyr 191 coordinates Fe^{3+} and Arg 121 are the synergistic anion binding residues. No sulphate binding to the dilysine trigger residues is observed, however, a water molecule is hydrogen bonded to Lys 209 and Tyr 191 and also to the sulphate ions in site 1 [23]. The homologous residue to the ovotransferrin Tyr 191 in hTf is Tyr 188 and it is likely that this residue will also have indirect links to bound sulphate. The only other apo-transferrin to be structurally characterised with anions bound is apo-lactotransferrin which shows chloride anions bound to the synergistic anion binding arginine residues in both the open N-lobe and closed C-lobe [24]. The chloride ions in apo-lactotransferrin interact directly with Tyr 192/528, the homologous residues with Tyr 188/517 in hTf. It is likely that apohTf will have similar sulphate binding sites to that found for the apo-ovotransferrin N-lobe [23]. The synergistic anion binding residues Arg 124/456, in particular are likely to be primary sulphate binding sites.

He et al. note that the lack of tyrosinate signal from the dilysine mutants of hTf/2N need not imply a lack of anion binding [19]. No negative absorption feature at 245 nm only means that Tyr 188 has a higher pK_a and does not report anion binding. Anion binding is likely to still occur in the same sites, though with different kinetics to the wild-type protein. Harris et al. studied the anion binding of the R124A, K206A, and K296A mutants apohTf/2N and found that all three mutants gave negative UV absorption difference peaks at 245 nm [11]. The kinetics of anion binding derived from the 245 nm feature for these mutants were indeed found to be different from the wild-type protein. The pH dependence of the 245 nm feature for all the hTf/2N mutants studied have not yet been reported. The X-ray structures of the K206A and K296A iron loaded structures have recently been solved and show a highly conserved hydrogen bonding network associated with the metal site with water molecules replacing the missing lysine ε-amino groups in the two mutants [25].

Interestingly, periodate-modified apohTf can still bind iron, but not with carbonate as the synergistic anion [26]. Periodate-modified apohTf create a dityrosine species between two of the iron binding or second shell tyrosine residues. Furthermore, the periodate-modified apohTf still exhibits a negative band at 245 nm upon addition of sulphate. It has been speculated that it is the iron binding tyrosines, Tyr 95/188 and Tyr 426/517, that are cross-linked to form the dityrosine species. The alignment of Tyr 95/85 and Tyr 426/412 in the closed form of hTf Fig. 6 are, however, much better suited for a 2,2′ linkage of the phenolic rings, which is more favoured in the radical reaction thought to be initiated by periodate. Theoretical studies also suggest that the presence of positively charged residues increase the probability of tyrosine coupling [27]. If Tyr 95/85 and Tyr 426/412 are 2,2′ covalently linked in periodate-modified apohTf, then this would leave Tyr 188 and Tyr 517 to act as electronic reporters of sulphate binding.

In conclusion, the UVRR spectra show that the binding of sulphate to apohTf results in the reprotonation of a tyrosine residue at pH 7.4. The pH dependency of tyrosinate UVRR signal reveals this tyrosine to possess an unusually low pK_a of 7.8. Previous work examining tyrosine hTf/2N mutants imply that Tyr 188 and Tyr 517 in apohTf are the residues with the low pK_a. The exact structural factors leading to Try 188 acquiring a low pK_a are not immediately apparent, though it appears that sulphate disrupts these factors by binding to the Arg 124/456 residues and other secondary sites in the interdomain cleft. The functional significance of this may be that with sulphate bound, both the binding of carbonate and the deprotonation of Try are disfavoured and as a result so is iron binding.

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