Spectrophotometric Evidence for Involvement of Aromatic Residues in the Interaction of Ferreascidin with Ferric Ion

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The iron(III)-binding properties of pyrocatechol and ferreascidin, a blood protein isolated from a stolidobranch ascidian (*Pyura stolonifera*), have been characterized by spectrophotometric titrations with metal ion in the presence of nitrilotriacetate (NTA) to maintain solubility under the conditions used (pH 7.0, I 0.2). Such studies of the model system have signified that the formation of the $[Fe(NTA)(pyrocatechol)]^{2-}$ complex is governed by a stability constant of $10^{32.5}$ M^{-2}. Corresponding studies of the interaction of iron(III) with the ascidian protein have provided spectrophotometric evidence for the coordination of each ferric ion to one tyrosine and two (3,4-dihydroxyphenyl)-alanine (DOPA) residues, the interaction being characterized by an effective stability constant of 4×10^{17} M^{-1} under the conditions studied (pH 7.0, I 0.2). Finally, a case is made for potential biological relevance of the iron(III)–ferreascidin interaction in the curing of this DOPA protein for the repair of wounds and/or securement of the ascidian to its substratum.

Introduction

Because iron salts form hydroxide precipitates under physiological conditions, the relatively high concentrations of iron that occur in the blood cells of a wide range of Ascidiacea[1-3] must reflect the presence of iron as metal chelates. In a search for the macromolecule responsible for such chelation of iron, an unusual protein has been isolated[4] from the blood cells of the stolidobranch ascidian *Pyura stolonifera*. The most characteristic feature of this 10 000-Da glycoprotein, named ferreascidin because of its strong affinity for Fe(III), is its extremely high content of aromatic residues (42% tyrosine, 17% (3,4-dihydroxyphenyl)alanine (DOPA), 8% phenylalanine).[4]

In this paper we examine the possibility that chelation of Fe(III) by ferreascidin involves aromatic residues of the protein. For quantitative characterization of the iron(III)-binding properties in terms of the strength of the interaction, the metal ion has usually been added in the presence of nitrilotriacetate (NTA) to maintain solubility under the conditions used (pH 7.0, I 0.2). Pyrocatechol is also titrated with the same iron(III)–NTA solution as a model system for the interaction with DOPA residues of ferreascidin.

Experimental Section

Materials. 2,2-Bis(hydroxymethyl)-2,2',2'-nitrilotriethanol (Bistris) and NTA (disodium salt) were used as supplied by Sigma Chemical Co. Iron(III) solutions were prepared from a ferric chloride solution (1 mg/mL Fe) supplied as an atomic absorption standard by BDH Chemicals Pty Ltd. *s*-Collidine was redistilled and pyrocatechol recrystallized from toluene prior to use. A stock solution (3.58 mM in Fe) was prepared by adding 1 equiv of ferric chloride to 2 equiv of NTA. After the addition of a further 1 equiv of NaOH, the pH was adjusted to 4.0 and the solution made to the required volume with distilled water.

Protein Purification. Ferreascidin was extracted and isolated from *P. stolonifera* as described previously,[4] most manipulations being carried out in a VAC drylab under nitrogen: buffers were outgassed by ultrasonication and purged with nitrogen. After the final purification step, the protein was concentrated by ultrafiltration (Amicon YM5 membrane) and then dialyzed against 5 mM Bistris buffer (pH 7.0) supplemented with 0.2 M NaCl in readiness for spectrophotometric titration with the iron–NTA solution. Concentrations of ferreascidin solutions were defined in terms of their DOPA contents, which were estimated as means of triplicate assays by an adaptation of the Arnow method for catechol.[5]

Spectrophotometric Titrations. Apoferreascidin or pyrocatechol (15–20 mL, 65–75 μM in terms of catechol) was transferred to a recycling system comprising a 25-mL reaction vessel with a quartz flow-cell (1- or 2-cm path length) and a peristaltic pump included in the return line to the reaction vessel, which was sealed under nitrogen by means of a Teflon lid fitted with a gastight septum. A 1-mL gastight syringe (SGE) filled with iron(III)–NTA solution was inserted into the septum to allow the addition of aliquots (40–50 μL) to the protein or pyrocatechol solution. Mixing was effected by constant circulation of the solution through the flow-cell assembly (2.5 mL/min) and by a magnetic stirrer located in the reaction vessel. After attainment of a constant absorbance reading at either 520 or 610 nm (3–5 min), the spectrum was recorded on a Hewlett Packard HP8540A diode array spectrophotometer. Difference spectra were generated by subtracting the spectrum of the apoprotein (or catechol) after each addition of iron(III), allowance being made for the effect of dilution. Throughout the titration, the temperature of the jacketed reaction vessel was maintained at 22 °C by circulation of water from a Colora Kryothermostat WK5.

Results and Discussion

Inasmuch as the major goal of the present investigation is to implicate DOPA residues in the chelation of ferric ion by ferreascidin, a logical starting point for these studies is the spectrophotometric characterization of complex formation between iron(III) and pyrocatechol. This approach has the advantage of providing insight into the likely spectral characteristics of iron–DOPA complexes and also of providing experience with allowance for the additional complications that arise from the necessity to add the ferric ion in the form of complexes with NTA.

Chelation of Fe(III) by Catechol in the Presence of NTA. In the present experiments catechol has been titrated with a solution comprising $FeCl_3$ and NTA in the stoichiometric molar ratio 1:2. Addition of this titrant to pyrocatechol in 5 mM Bistris–0.2 M

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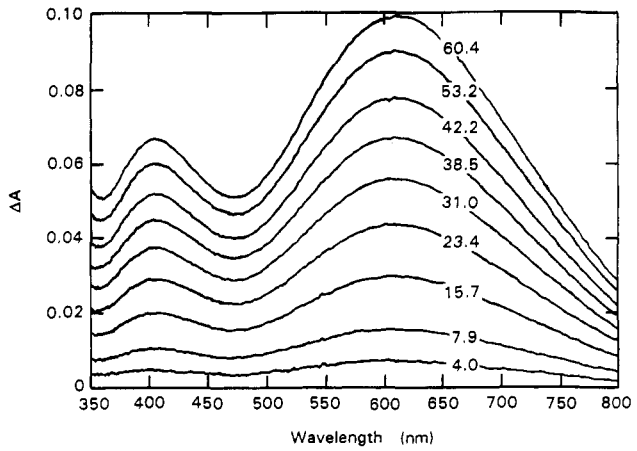

Figure 1. Difference spectra obtained in the titration of catechol (71.5 μM) in 5 mM Bistris–0.2 M NaCl (pH 7.0) with iron–NTA solution. Numbers denote the concentration (μM) of the titrant ($[Fe]_t$) giving rise to the various spectra.

NaCl, pH 7.0, gives rise to a blue complex with absorption maxima in the visible region at 404 and 610 nm (Figure 1). These ligand-to-metal charge-transfer peaks correspond closely with those of 410 and 618 nm reported[6] for the $[Fe(NTA)cat]^{2-}$ species in methanol. Indeed, the slightly lower λ_{max} values observed in Figure 1 can be attributed to the blue shift that occurs[6] on a change to a more polar solvent than methanol.

Further confirmation that the complex contains iron and catechol in a 1:1 ratio was obtained by Job's method of continuous variation.[7] Figure 2 presents the dependence of the absorbance at 610 nm upon the molar proportion of catechol (x) in mixtures with a value of 0.29 mM for the summed concentrations of iron (present mainly as nitrilotriacetato complexes) and catechol. The position of the peak is certainly consistent with the formation of a 1:1 complex and seemingly precludes the possibility[8,9] that bis(catecholato)iron(III) complexes form to any significant extent under the present conditions (pH 7.0, I 0.2). In that regard, we note that similar evidence of 1:1 stoichiometry has been obtained[10] for the Fe(III)–NTA–pyrocatechol-3,5-disulfonate system at pH 5.4 and that calculations based on the reported stability constants for the Fe(III)–NTA–catechol system also point to the virtual absence of the $[Fe(cat)_2]^-$ species in the range of titrant concentrations (4–180 μM Fe(III), 8–360 μM NTA) covered by the present experiments.

Since the spectral characteristics are those of a complex with iron, NTA, and catechol in equimolar proportions, the evaluation of a ligand-binding constant for the interaction is reliant upon determination of the concentration of Fe(NTA) in mixtures. Initially, the only concentrations of known magnitude are those of total catechol ($[cat]_t$), total iron ($[Fe]_t$), and total nitrilotriacetate ($[NTA]_t$). However, the concentrations of iron and NTA complexed with catechol may be evaluated as $(\Delta A/\Delta A_m)[cat]_t$, where $\Delta A/\Delta A_m$, the absorbance difference at λ_{max} for a given titrant concentration as a proportion of maximal change, also expresses the fraction of catechol chelated to Fe(NTA). There now remains the problem of considering the states of the rest of the Fe(III) and NTA.

Stability constants of $10^{15.9}$ M^{-1} (β_1) and $10^{24.3}$ M^{-2} (β_2) have been reported[11,12] for Fe(NTA) and $[Fe(NTA)_2]^{3-}$ complexes,

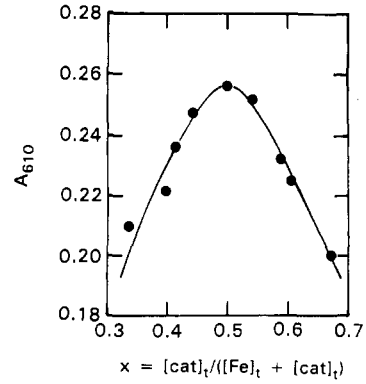

Figure 2. Determination of the iron:catechol stoichiometry for the Fe(III)–NTA–catechol interaction by Job's method. The abscissa denotes the molar proportion of catechol in mixtures with a combined total concentration of 0.29 mM.

respectively. Those values refer to the situation with nitrilotriacetate in a fully deprotonated state and therefore need to be modified to take into account the state of NTA protonation under the conditions of the experiment. At pH 7.0, nitrilotriacetate is essentially a divalent anion, $HNTA^{2-}$, and accordingly

$$\beta_i^{eff} = \beta_i/(\alpha_L)^i \qquad i = 1 \text{ or } 2 \tag{1a}$$

$$\alpha_L = 1 + K^H_{HNTA^{2-}}[H^+] \tag{1b}$$

where β_i^{eff} is the effective stability constant and $K^H_{HNTA^{2-}}$, the association constant for the final proton ionization on nitrilotriacetic acid, may be taken[13,14] as $10^{9.73}$. On this basis, the effective stability constants at pH 7.0 become 1.48×10^{13} M^{-1} and 6.89×10^{18} M^{-2} for β_1^{eff} and β_2^{eff}, respectively. An expression for the total concentration of ferric ion, $[Fe]_t$, may now be written[11] as

$$[Fe]_t = (\Delta A/\Delta A_m)[cat]_t + [Fe^{3+}](\beta_1^{eff}\alpha_{MX}[NTA] + \beta_2^{eff}[NTA]^2) \tag{2a}$$

$$\alpha_{MX} = 1 + 1/(K^H_{MX}[H^+]) + 1/(K^H_{MX}K^H_{MXOH}[H^+]^2) \tag{2b}$$

where [NTA] denotes the concentration of uncomplexed nitrilotriacetate (without regard to its protonation state) and where α_{MX} takes into account the fact that the Fe(NTA) complex (MX) undergoes two deprotonation steps that are governed by association constants K^H_{MX} and K^H_{MXOH} ($10^{4.08}$ and $10^{7.77}$ M^{-1}, respectively): α_{MX} is equivalent to β_{III} in Schwarzenbach terminology.[11] The absence from eq 2a of a term for chelation of Fe^{3+} by the 5 mM Bistris buffer has been justified experimentally by establishing the similarity of binding data (Figure 3) from experiments in which Bistris was replaced by 5 mM *s*-collidine, a compound whose ability to form metal complexes is sterically restricted by the presence of methyl groups next to the pyridine N.

The corresponding expression for total nitrilotriacetate is given by

$$[NTA]_t = (\Delta A/\Delta A_m)[cat]_t + [NTA](1 + \beta_1^{eff}\alpha_{MX}[Fe^{3+}] + 2\beta_2^{eff}[Fe^{3+}][NTA]) \tag{3}$$

from which it is possible to eliminate terms in $[Fe^{3+}]$ by writing them in terms of total iron concentration (eq 2): the fact that $[NTA]_t = 2[Fe]_t$ is also incorporated at this stage. Rearrangement of the expression so obtained then allows [NTA], the concentration of free nitriloacetate, to be obtained as the positive

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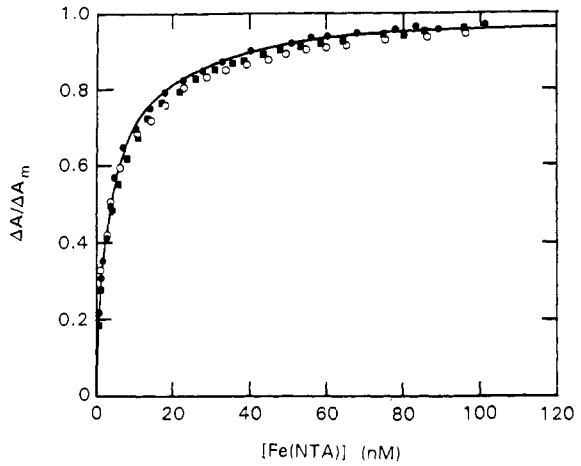

Figure 3. Binding curves obtained by difference spectroscopy at 610 nm for the interaction of Fe(NTA) with catechol: (●,■) results from duplicate titrations of catechol in 5 mM Bistris–0.2 M NaCl (pH 7.0); (○) results from a titration in 5 mM *s*-collidine–0.2 M NaCl (pH 7.0). The solid line is the best-fit theoretical relationship obtained by analysis of the data in terms of eq 5.

root of the quadratic

$$\beta_2^{\text{eff}}[\text{NTA}]^2 + \{\beta_1^{\text{eff}}\alpha_{\text{MX}} - \beta_2^{\text{eff}}(\Delta A/\Delta A_m)[\text{cat}]_t\}[\text{NTA}] - \beta_1^{\text{eff}}\alpha_{\text{MX}}[\text{Fe}]_t = 0 \quad (4)$$

After calculation of the corresponding free concentration of ferric ion, $[Fe^{3+}]$, from eq 2a, the concentration of Fe(NTA) in each mixture then follows as the product of β_1^{eff}, [NTA], and $[Fe^{3+}]$. On the grounds that catechol possesses a single site for Fe(NTA), the apparent binding constant for the Fe(NTA)–catechol interaction, $(K_{\text{FeNTA}}^{\text{cat}})_{\text{app}}$, may then be obtained from the rectangular hyperbolic relationship

$$\Delta A/\Delta A_m = (K_{\text{FeNTA}}^{\text{cat}})_{\text{app}}[\text{FeNTA}]/\{1 + (K_{\text{FeNTA}}^{\text{cat}})_{\text{app}}[\text{FeNTA}]\} \quad (5)$$

In order to avoid the error distortion inherent in linear transforms of the rectangular hyperbolic binding expression, the untransformed ($\Delta A/\Delta A_m$, [Fe(NTA)]) results (Figure 3) have been analyzed directly in terms of eq 5. The apparent association constant of 2.5 (±0.7) × 10^8 M^{-1} so obtained refers to the interaction of Fe(NTA) with catechol that is essentially fully protonated. Conversion to a conventional stability constant on the basis that the protonation of catechol is governed[9] by constants of $10^{9.2}$ and $10^{13.0}$ leads to a value of $10^{32.5}$ M^{-2} for $\beta = [\text{Fe(NTA)cat}]^{2-}/([Fe^{3+}][\text{NTA}^{3-}][\text{cat}^{2-}])$. In that regard, Morin and Scharff[8] report a stability constant of $10^{30.8}$ M^{-2} for the interaction of ferric ion, NTA, and pyrocatechol-3,5-disulfonate; and the same value may be inferred from the apparent association constant of 1.22 × 10^4 M^{-1} at pH 5.4 that was reported by Schwarzenbach and Willi.[10]

Spectral Features of the Fe(III)–Ferreascidin Interaction. Although preliminary reports from this laboratory[4,15] have suggested that ferreascidin binds two atoms of iron, no firm conclusion about the stoichiometry was then possible. This indecision stems from microheterogeneity of different preparations that is manifested as a variation in the relative absorbances of spectral peaks at 277 and 360 nm. Fortunately, the interaction of iron(III) with ferreascidin is seemingly independent of this microheterogeneity insamuch as a unique characterization of the chelation phenomenon can be effected by expressing reactant concentrations of protein in terms of DOPA content. Furthermore, EPR spectra of iron(III)–ferreascidin complexes exhibit the characteristics $g = 4.24$ resonance that typifies a high-spin iron(III) in an orthorhombic environment. The binding of Fe(III) to ferreascidin must therefore occur in such a manner that no two iron atoms are located within a common domain of influence: independent binding may therefore be assumed.

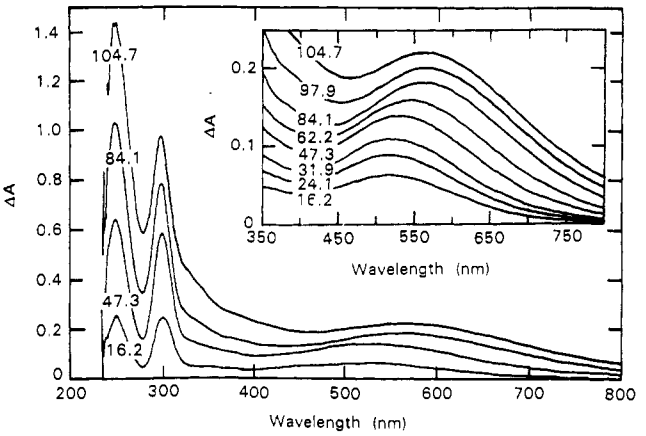

Figure 4. Difference spectra obtained in the titration of ferreascidin (66.3 μM DOPA) in 5 mM Bistris–0.2 M NaCl (pH 7.0) with iron–NTA solution. Numbers denote the concentration (μM) of the titrant ($[Fe]_t$) giving rise to the various spectra. Inset: detail of difference spectra in the visible region showing the drift of the maximum from 520 to 570 nm at high titrant concentrations.

The interaction of ferreascidin (66.3 μM DOPA) in 5 mM Bistris–0.2 M NaCl (pH 7.0) with a range of concentrations of iron(III)–NTA solution is followed spectrophotometrically in Figure 4. The first point to note is that complex formation at low titrant concentrations is characterized by the development of an absorption peak at 520 nm but that at higher concentrations (>40 μM Fe) its position drifts toward 570 nm, the λ_{max} for the bis(catecholato)iron(III) complex.[9] Use of $FeCl_3$ as titrant leads to spectra in which this developing chromophore remains at 510–520 nm (data not shown). Secondly, with either titrant, pairs of chromophores are also observed in the UV region of the spectra (247–250 and 296–299 nm), reflecting perturbation in the $\pi \rightarrow \pi'$ transitions of aromatic rings associated with chelation of phenolic oxygens to metal ions.[16] Evidence that the developing chromophores at 296 and 520 nm are both consequences of the same Fe(III)–ferreascidin interaction was obtained by establishing the identity of $[Fe]_t$ dependences of the fractional saturation, $\Delta A/\Delta A_m$, at the two wavelengths (data not shown). Inasmuch as tyrosine may also be involved in complex formation between Fe(III) and ferreascidin, it is of interest to note that the binding of phenolate causes a shift of 2000 cm^{-1} to higher energy in the ligand-to-metal charge transfers of a variety of iron(III) complexes.[17] On this basis the incorporation of one tyrosine into the coordination sphere of bis(catecholato)iron(III) would shift λ_{max} of the latter from 570 to 512 nm, which is close to the value of 520 nm observed for ferreascidin (Figure 4).

Having made a case for considering the developing chromophore at 520 nm to reflect formation of an iron(III)–ferreascidin complex with metal chelated to one tyrosine and two DOPA residues, we now need to rationalize the subsequent shift of this peak to 570 nm during the latter stages of the titration with Fe(III)–NTA solution (Figure 4). The fact that 570 nm corresponds to the bis(catecholato)iron(III) chromophore[9] points to the likelihood of tyrosine displacement from the complex by high NTA concentrations, this being a conclusion that is consistent with the failure to observe the spectral shift in experiments with $FeCl_3$ as titrant. However, any such explanation must first be reconciled with the continued increase in absorbance associated with the

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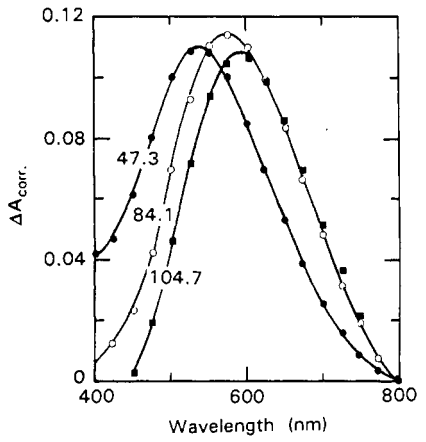

Figure 5. Difference spectra, corrected for light scattering, obtained in the titration of ferreascidin in 5 mM Bistris–0.2 M NaCl (pH 7.0) with iron–NTA solution. Numbers denote the concentrations (μM) of titrant ($[Fe]_t$) giving rise to the various spectra, the uncorrected versions of which are shown in Figure 4.

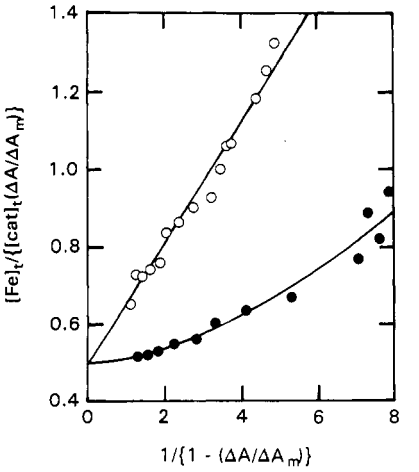

Figure 6. Evaluation of the Fe:DOPA stoichiometry for the interaction of ferreascidin in 5 mM Bistris–0.2 M NaCl (pH 7.0) with iron–NTA solution (●) and $FeCl_3$ (○), the results being plotted in accordance with eq 7.

spectral shift (Figure 4). We therefore examine the extent to which the change in ΔA may be attributed to increased light-scattering in the latter part of the titration.

By application of the Leach and Scheraga procedure[18] to the spectrum of apoferreascidin, it was found that the light-scattering contribution to absorbance at a given wavelength, ΔA^s_λ, could be described by the relationship

$$\log \Delta A^s_\lambda = \log \Delta A^s_{800} - 2.0(\log \lambda - \log 800) \qquad (6)$$

where the reference position (800 nm) was selected on the grounds that all apparent absorption at this wavelength should be attributable to turbidity. Use of eq 6 to make allowance for light-scattering effects in spectra from the latter part of the titration of ferreascidin with iron(III)–NTA solution showed that absorption of the chromophore in the visible region does, indeed, remain essentially constant during the shift in λ_{max} from 520 to 570 nm (Figure 5). Such behavior is entirely consistent with the proposition that higher concentrations of NTA cause displacement of tyrosine from a ferreascidin–Fe(III) complex in which the metal ion is chelated to two DOPA residues and a tyrosine,[19] in which case the formation of that complex may be characterized without recourse to data reflecting the spectral shift associated with the subsequent tyrosine elimination.

Stoichiometry of the Fe(III)–Ferreascidin Interaction. The next step toward quantitative characterization of the ferreascidin–Fe(III) interaction entails delineation of the reaction stoichiometry. In that regard, the spectral data signify the formation of complexes with iron(III) and DOPA in a 1:2 ratio, but there is no guarantee that all DOPA residues of ferreascidin are necessarily available for interaction with iron. What is required, therefore, is an estimate of the overall stoichiometry in terms of iron(III) bound per mole of DOPA, the basis on which ferreascidin concentrations have been defined. For this purpose, advantage is taken of the expression[20]

$$[Fe]_t/[[cat]_t(\Delta A/\Delta A_m)] = p + 1/\{K[cat]_t[1 - (\Delta A/\Delta A_m)]\} \qquad (7)$$

in which p denotes the number of sites for iron(III) per DOPA residue and $[cat]_t$ the total ferreascidin concentration expressed on a DOPA (catechol) basis. However, in so doing, it must be recognized that the existence of iron(III) in a variety of NTA-chelated states may negate an assumption, inherent in eq 7, that a single intrinsic association constant (K) describes the ferreascidin–Fe(III) interaction when metal ion concentration is expressed solely in terms of $[Fe]_t$. Nonfulfillment of that condition may well lead to nonlinear dependence of $[Fe]_t/[[cat]_t(\Delta A/\Delta A_m)]$ upon $1/[1 - (\Delta A/\Delta A_m)]$, but the curvilinearity should not influence unduly the estimation of the reaction stoichiometry, p, from the ordinate intercept of such a plot. This inference is borne out by graphical analysis of the spectral data for ferreascidin (corrected for light-scattering) in terms of eq 7 (Figure 6), which indicates a stoichiometry of one Fe atom per two DOPA residues ($p = 0.5$) for reaction mixtures with $FeCl_3$ (○) and Fe(III)–NTA solution (●) as titrant.[21] The important conclusion is therefore reached that all Arnow-reactive DOPA residues in ferreascidin are accessible for chelation with Fe(III).

Characterization of the Fe(III)–Ferreascidin Interaction. The final difficulty to be overcome in quantitative characterization of the iron(III)–protein interaction is identification of the reaction stoichiometry in the presence of NTA. Because one tyrosine and two DOPA residues account for five of the six coordination sites on Fe(III), the possibility must clearly be entertained that $[Fe(H_2O)_6]^{3+}$ is the metal ion species that is complexed by ferreascidin. On the other hand, Fe(NTA) cannot be excluded as a possible candidate in view of its involvement in the interaction with catechol (Figure 3). A second situation is therefore envisaged in which the interaction of Fe(NTA) with the five ferreascidin sites is accompanied by dissociation of the quadridentate NTA molecules, and a third, in which the NTA remains attached to the sixth coordination position on the Fe(III) ion. It transpires that the first two of these three situations are thermodynamically equivalent, a factor evident from comparison of expressions for the two equilibrium constants. If $[Fe(H_2O)_6]^{3+}$ is regarded as the specific metal ion species undergoing complex formation with ferreascidin, the expression for the equilibrium constant may be written as

$$K_{Fe(III)} = \frac{\Delta A/\Delta A_m}{[1 - (\Delta A/\Delta A_m)][Fe^{3+}]} \qquad (8)$$

whereas that for the exchange reaction involving elimination of

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(19) Unfortunately, corresponding attempts to observe this release of tyrosine as a decrease in the magnitude of the difference peak at 296 nm were thwarted by contributions of Fe(III)–NTA complexes to absorbance in this wavelength region at the titrant concentrations required to effect the displacement.

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(21) The more curvilinear form of the plot for the system with an Fe(III)–NTA solution as titrant reflects the change in NTA concentration associated with change in $[Fe]_t$ and hence a dependence of the relative proportion of chelating species, Fe^{3+} or Fe(NTA), upon $[Fe]_t$.

NTA is

$$K_{ex} = \frac{(\Delta A/\Delta A_m)[NTA]}{[1-(\Delta A/\Delta A_m)]\beta_1^{eff}[Fe^{3+}][NTA]} = K_{Fe(III)}/\beta_1^{eff} \quad (9)$$

It is immaterial which of these two situations is considered, because the equilibrium constant for the other follows directly from that for the case examined. We shall therefore proceed with analyses on the basis of only two models: one in which $[Fe(H_2O)_6]^{3+}$ is the interacting species and the other in which the binding of Fe(NTA) to ferreascidin does not entail the elimination of NTA.

For the model with Fe(NTA) as the active iron(III) species, the expressions for mass conservation of iron and nitrilotriacetate (analogous to eqs 2 and 3 and incorporating the identity $[NTA]_t = 2[Fe]_t$ in these experiments) become

$$[Fe]_t = (\Delta A/\Delta A_m)[cat]_t/2 + [Fe^{3+}](\beta_1^{eff}\alpha_{MX}[NTA] + \beta_2^{eff}[NTA]^2) \quad (10a)$$

$$[NTA]_t = (\Delta A/\Delta A_m)[cat]_t/2 + [NTA](1 + \beta_1^{eff}\alpha_{MX}[Fe^{3+}] + 2\beta_2^{eff}[Fe^{3+}][NTA]) \quad (10b)$$

As before, the free concentration of NTA may be obtained from these two equations as the solution of a quadratic, namely

$$\beta_2^{eff}[NTA]^2 + [\beta_1^{eff}\alpha_{MX} - \beta_2^{eff}(\Delta A/\Delta A_m)[cat]_t/2][NTA] - \beta_1^{eff}\alpha_{MX}[Fe]_t = 0 \quad (10c)$$

Alternatively, consideration of $[Fe(H_2O)_6]^{3+}$ to be the active iron species still gives rise to eq 10a for the conservation of iron, but the remaining two expressions are

$$[NTA]_t = [NTA](1 + \beta_1^{eff}\alpha_{MX}[Fe^{3+}] + 2\beta_2^{eff}[Fe^{3+}][NTA]) \quad (11a)$$

$$\beta_2^{eff}[NTA]^2 + [\beta_1^{eff}\alpha_{MX} - \beta_2^{eff}(\Delta A/\Delta A_m)[cat]_t][NTA] - \beta_1^{eff}\alpha_{MX}[[Fe]_t + (\Delta A/\Delta A_m)[cat]_t/2] = 0 \quad (11b)$$

In either instance the concentration of free ferric iron, $[Fe^{3+}]$, may then be calculated, and hence the appropriate binding curve, constructed.

Figure 7a summarizes the binding data obtained on the basis that Fe(NTA) is the active species and also the rectangular hyperbolic relationship (—) corresponding to the mean of constants obtained by analysis of each experimental point in terms of eq 5. On the grounds that the poor description of the experimental results by the theoretical relationship signifies systematic rather than random deviations from the mean equilibrium constant (0.65×10^9 M^{-1}), it may be concluded that the reaction under scrutiny in Figure 7 is not simply the addition of Fe(NTA) to ferreascidin. We therefore turn to the corresponding analysis of the same spectral results with $[Fe(H_2O)_6]^{3+}$ as the active agent in the interaction with ferreascidin (Figure 7b). For this model the mean rectangular hyperbolic relationship (—) clearly provides a much better description of the experimental results. However, although this greater conformity between theory and experiment indicates compliance of the Fe(III)–ferreascidin system with a model in which the interaction of protein with $[Fe(H_2O)_6]^{3+}$ is governed by an effective intrinsic association constant of 4.1 (±0.7) $\times 10^{17}$ M^{-1}, this result is also consistent with interpretation in terms of the exchange reaction involving Fe(NTA) and the elimination of NTA. Indeed, from eq 9 the magnitude of the equilibrium constant for the exchange reaction would be 2.8 (±0.4) $\times 10^4$. From the thermodynamic viewpoint, the former description is the equivalent of a stability constant but differs therefrom in that it refers to the interaction at pH 7 rather than to the situation in which the DOPA and tyrosine residues are in deprotonated states.

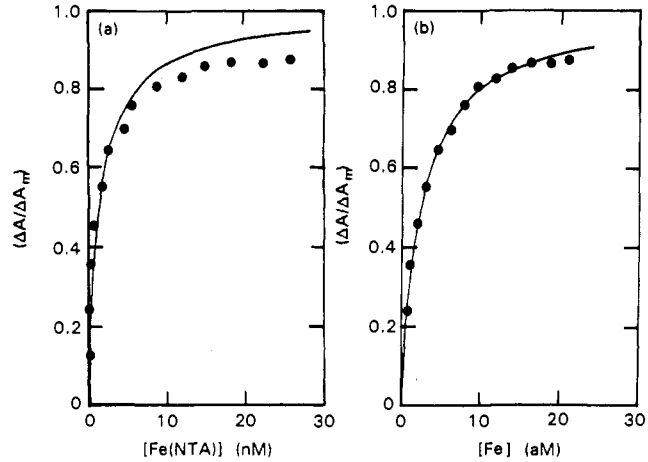

Figure 7. Characterization of the interaction of ferreascidin in 5 mM Bistris–0.2 M NaCl (pH 7.0) with iron–NTA solution: (a) comparison of the data obtained on the basis of Fe(NTA) as the active agent with the attempted theoretical curve fit (—) to a rectangular hyperbolic relationship with unity as the upper limit for $\Delta A/\Delta A_m$; (b) corresponding comparison on the basis that Fe^{3+} is the chelating agent.

Concluding Remarks

From the chemical viewpoint, the major outcome of this investigation into the iron(III)-binding properties of ferreascidin at pH 7.0 has been the provision of spectrophotometric evidence for the coordination of each ferric ion to one tyrosine and two DOPA residues. On the basis that all Arnow-reactive DOPA residues have been shown to be accessible for chelation with iron(III), the earlier report[15] of a binding capacity of two iron atoms per ferreascidin molecule may be an underestimate of the stoichiometry inasmuch as there are seemingly more than four DOPA residues in the 10 000-Da glycoprotein.[4] The interaction is described by an effective stability constant of 4×10^{17} M^{-1} under the present conditions (pH 7.0, I 0.2).

The iron(III)-binding characteristic of ferreascidin has potential physiological implications in the histology of *P. stolonifera*, and of ascidians in general. In the search for the ligands responsible for the sequestration of metal ions from seawater, several polypeptides, including the tunichromes[22] and halocyamines,[23] have been suggested as fulfillers of this role in vanadium- and iron-accumulating ascidians. All are structurally related in that they contain DOPA, (3,4,5-trihydroxyphenyl)alanine (TOPA), or α,β-unsaturated derivatives thereof. Ferreascidin is clearly another protein/polypeptide with similar potential; but, in keeping with the situation for the tunichromes and halocyamines, there is still no evidence for the existence of the metal–protein chelate in cells, even though the formation of complexes can be demonstrated in vitro. Thus, whereas the iron(III)–ferreascidin complex is purple at neutral or alkaline pH, intact morula cells of *P. stolonifera* are yellow.

In the absence of evidence for a role of ferreascidin in the sequestration of iron(III) in the ascidian blood cells, we turn to the possibility that the Fe(III)–ferreascidin interaction may have implications in the blood cell chemistry of *P. stolonifera* after cell lysis. In that regard Endean[1] has reported that the addition of dilute NaOH to the corpuscles of this ascidian produced a "transient purplish gelatinous mass". Given the fact that the blood cells contain both ferreascidin and iron, it is highly probable that the reported material comprised mainly the complex described

(22) Oltz, R. C.; Bruening, R. C.; Smith, M. J.; Kustin, K.; Nakanishi, K. J. *J. Am. Chem. Soc.* **1988**, *110*, 6162.
(23) Azumi, K.; Yokosawa, H.; Ishii, S. *Biochemistry* **1990**, *29*, 159.

in this study. Furthermore, the Endean finding[1] may be relevant to the biological context, where the slightly alkaline environment of seawater could well cause blood cells at the surface of an injury to lyse and form the Fe(III)–ferreascidin complex. In line with the suggestion that metal chelation is part of the quinone tanning process in DOPA proteins,[24] we propose that the presence of a storehouse of iron(III) in the blood cells of *P. stolonifera* may reflect a role of the Fe(III)–ferreascidin interaction in curing this DOPA protein for the repair of wounds and/or for securement of the ascidian to its substratum.

(24) Waite, J. H. *Comp. Biochem. Physiol.* **1990**, *97B*, 19.

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