pH Dependence of the enantioselective excited-state quenching of L,D-lanthanide(III) tris (pyridine-2,6-dicarboxylate) chelates by ferricytochrome c from horse heart and ferricytochrome c-550 from Paracoccus versutus
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pH dependence of the enantioselective excited-state quenching of \( \Lambda, \Delta\)-Tb(III) and \( \Lambda, \Delta\)-Eu(III)tris(pyridine-2,6-dicarboxylate) chelates by ferricytochrome \( c \) from horse heart and ferricytochrome \( c \)-550 from \( Paracoccus \) \( versutus \)

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Abstract The pH dependence of the dynamic quenching of the luminescence from Tb(III) and Eu(III) tris(pyridine-2,6-dicarboxylate) chelates by the title proteins is studied. For Tb(DPA)\(_3^+\) also the quenching by the Lys 14\( \rightarrow \)Glu and Lys99\( \rightarrow \)Glu mutants of cytochrome \( c \)-550 (cytc-550) is investigated. The rate constants for quenching of the electronically excited \( \Lambda \) and \( \Delta \) enantiomers of the luminophore by equine cytochrome \( c \) show a sharp decrease upon increasing the pH from 7 to 10, which can be described phenomenologically by deprotonation of a single acidic group with \( pK_a \) of 9.2 \( \pm \)0.1 for Eu and 9.4 \( \pm \)0.1 for Tb. These values are similar to that found for the alkaline transition of the protein. The alkaline conformer(s) of the protein at pH\( \geq 10 \) is found to be a very inefficient quencher of the lanthanide luminescence. For Tb, but not for Eu, a significant lowering of the degree of enantioselectivity (\( E_q \)) in the quenching is found along with a reduction of the quenching rates. For cytc-550, the decrease of the quenching rate constants with increasing pH is described by \( pK_a = 9.8 \pm 0.1 \) and for the two mutants the same value is obtained. For the cytc-550 proteins the change of the quenching rates at high pH is ascribed to a reduction of the binding affinity of the excited lanthanide complex to the surface area of the protein near the exposed heme edge, caused by deprotonation of (presumably) several lysine residues.

Key words Cytochrome \( c \) · Cytochrome \( c \)-550 · Alkaline transition · Enantioselective luminescence quenching · Lanthanide complexes

Abbreviations \( cytc \) cytochrome \( c \) from horse heart · \( cytc-550 \) cytochrome \( c \)-550 from \( Paracoccus \) \( versutus \) · \( wt \) wild type

Introduction

Enantioselective quenching by \( c \)-type cytochromes

Many proteins show a remarkable enantioselectivity towards substrates in their enzymatic activity. This chiral discrimination is of biological and pharmacological importance and, moreover, fascinating from a chemical point of view. We have recently reported on enantioselectivity displayed by \( c \)-type cytochrome proteins in their quenching of the luminescence from enantiomeric lanthanide complexes [1]. In certain respects this quenching reaction can be regarded as a model system for chiral discriminatory protein-ligand interactions, allowing the reaction rates and enantioselectivity to be determined with time-resolved emission spectroscopy. With this method also the ionic strength [1], temperature and pressure dependence [2] of the reaction can be investigated.

The proteins studied are ferricytochrome \( c \)-550 from \( Paracoccus \) \( versutus \)\(^1\) (cytc-550) and ferricytochrome \( c \) from horse heart (cytc). A small quantity of protein (typical concentration 10 \( \mu \)M) is added to an aqueous solution containing racemic \( (\Lambda,\Delta)\)Ln(III) tris(pyridine-2,6-dicarboxylate) chelate (\( \sim 1 \) mM). We abbreviate Ln(DPA)\(_3^+\) by Ln (\( = \)Tb or Eu). The addition results in a shortening of the lifetime of the lanthanide in the excited state (denoted by Ln*), and thus in a

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\(^1\) This bacterium was previously referred to as \( Thiobacillus \) \( versutus \) [3]
quenching of the luminescence which is ascribed to
electronic energy transfer from the electronically ex-
cited lanthanide to the heme group of the protein. Ow-
ing to enantioselectivity in this process, the rate con-
stants for quenching of \( A-Ln^* \) and \( A-Ln^* \) are different and the lifetimes of the two excited state populations
are not equal any more. The overall reaction can be de-
scribed by a pre-equilibrium step involving the forma-
tion of an encounter complex in which \( Ln^* \) is bound to
the protein (association constant \( K^s \), where \( s \) denotes
the sense of chirality of \( Ln^* \) or \( A \)), followed by the
energy transfer step (rate constant \( k^T \)).

A characteristic structural feature of the \( c \)-type cy-
tochromes is that one edge of the heme group is ex-
posed to the solvent. As the electronic interactions
leading to energy transfer between the energy donating
and accepting groups strongly decay with distance \( R
(\alpha R^{-n}, n \geq 6) \), energy transfer occurs predominantly
from excited lanthanide chelates bound at the protein’s
surface close to the solvent exposed heme edge. Bind-
ing of the triply negative charged \( Ln \) species is electro-
statically favoured by the presence of several positively
charged lysine residues around this edge [1]. For cytc-
550, specific mutation of either one of two lysine resi-
dues near the exposed heme edge, Lys14 or Lys99, to a
glutamate (to yield K14E and K99E cytc-550) results in
a considerable reduction of the quenching rate. More-
over, mutation of Lys99 also affects the enantioselectiv-
ity in the quenching. These observations, together with
theoretical considerations, have led us to propose a ten-
tative geometry for the two diastereomeric \( Ln \)-cytc-550
complexes in which the energy is transferred. In both
complexes, two carboxylate oxygen atoms (each on a
different DPA ligand) form hydrogen bonds with pro-
tons on the terminal nitrogen atoms of Lys99 and
Lys54. The pyridine ring of one ligand interacts with
Phe53 [1].

Because of the large enantioselectivity in the
quenching reaction and its sensitivity for alterations in
the protein structure, we have suggested that the enan-
tioselective quenching can be used as a probe for the
protein structure around the exposed heme edge. In the
present work, the effect of \( pH \) on the enantioselective
quenching by cytc and cytc-550 is studied, since it is
known that at alkaline \( pH \) a conformational transition
occurs in the protein in which the structure near the
heme group is affected.

The alkaline transition

Many ferricytochrome \( c \) proteins undergo a confor-
entional change at \( pH > 7 \), the so-called alkaline transition
[4]. The alkaline conformer of cytc (i.e. the species
present at \( pH > 10 \) ) has a drastically altered redox po-
tential compared to the native conformer and is no
longer reduced by ascorbate, Fe(CN)\(_6\)\(^{4-}\) or ferrocyto-
chrome \( c1 \), a biological redox partner (see, e.g. [5]).
Also its electronic absorption spectrum differs: at high
\( pH \) the 695 nm absorption band of moderate intensity
(\( \varepsilon_{\text{max}} = 10^3 \text{ cm}^{-1} \text{ M}^{-1} \)) disappears from the spectrum.
The alkaline transition has been characterized by se-
veral other experimental techniques as well (see, e.g.
[6]). Despite the extensive characterization, the un-
derlying molecular details of the alkaline transition are
still a matter of debate, although it is now commonly
agreed upon that a main aspect is the replacement of
Met80 by a nitrogen base (probably a deprotonated ly-
sine) as the sixth ligand of the central iron atom. The
observation of several sets of heme methyl NMR sig-
als at high \( pH \) indicates that several conformers of the
protein exist and that more than one amino acid side-
chain can act as a ligand of the iron atom.

The alkaline transition of several ferricytochrome \( c \)
proteins has been investigated: in yeast iso-1-cytc and
horse heart cytc it involves the abstraction of one pro-
ton characterized by a \( pK_a \) value of \( \approx 8.5 \) and \( 9-9.4 \), re-
spectively [4]. For \( P. \) versutus ferricytc-550, a \( pK_a \) value
of 11.2 has been reported [7, 8]. For this protein, the
change in the absorption spectrum upon increasing \( pH \)
is not exactly that expected for a one-proton titration,
but more complex. In the case of yeast iso-1-cytc, it
could be shown that Lys79 [9] and Lys73 [10] can both
act as a ligand at high \( pH \). For cytc-550, Ubbink et al.
have shown that neither Lys99 [11] (which is homolo-
gous to Lys79 in yeast iso-1-cytc and horse cytc) nor
Lys14 [8] (homologous to Lys13 in horse cytc) serve as
a ligand of the iron atom [12].

In this study the \( pH \) dependence of the rates and en-
antioselectivity of the quenching of Eu- and
\( \text{Tb} \)(DPA)\(_3\)\(^{3-}\) luminescence will be investigated. For this
study, cytc and cytc-550 were chosen because their
structure shows considerable similarity, but the \( pH \) at
which the alkaline conformation occurs differs for both
proteins. This allows one to look for a correlation be-
tween changes in the quenching as a function of \( pH \)
and the conformational transition of the protein. For \( \text{Tb} \)
luminescence the quenching by two site-specifically
mutated cytc-550 proteins (K14E and K99E) will be in-
vestigated as well. These experiments may reveal to
what extent the ionization of a single residue can affect
the \( pH \) dependence of the energy transfer reaction.

Materials and methods

The purification of horse heart cytc (Sigma Chemicals) has been
described previously [1]. The isolation and purification of the \( P.
versutus \) wt cytc-550 [13], K14E cytc-550 [8] and K99E cytc-550
[11] proteins from \( E. \) coli were performed as described.

Preparation of samples

The Ln(DPA)\(_3\)\(^{3-}\) complexes were prepared as described earlier
[14]. Protein concentrations were determined spectrophotometri-
cally, using \( 8.4 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} \) [15] and \( 10.8 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1} \) [7]
as values for the extinction coefficients at 550 nm of oxidized cy-
t and oxidized cytc-550, respectively.
Measurements

The time-resolved luminescence and circular polarization luminescence (CPL) measurements were performed on a home-built photon-counting spectrometer [16]. All measurements were carried out in aqueous solutions at room temperature (20±1°C). At pH > 9, c-type ferricytochrome protein solutions are not completely stable: they tend to undergo autoreduction. This was prevented by adding small quantities of K₃Fe(CN)₆. The results obtained with such samples were the same as measured in the absence of oxidant. Fe(CN)₆₃⁻ does not measurably quench the Tb* or Eu* emission.

Determination of the enantioselectivity in the quenching

The decay constants for the excited state population of the Δ and Λ enantiomers of the Ln(DPA)₃⁻ complex, k⁺ and k⁻, may be obtained by fitting the biexponential function (Eq. 1) to the experimental decay trace [14]. In the fit, the number of dark counts is included as an instrumental parameter.

\[ I(t) = I₀ [\exp(-k⁺t) + \exp(-k⁻t)] + (\text{dark counts}) \]  \hspace{1cm} (1)

From the decay constants the quenching rate constants, k⁺ and k⁻, can be calculated using Eq. 2:

\[ k_\pm = k_0 + k_\pm [Q] \] \hspace{1cm} (2)

where k₀ is the decay constant in the absence of the quencher Q. The degree of enantioselectivity is defined as

\[ E_q = \frac{(k⁺ - k⁻)(k⁺ + k₀ + k₀)}{(k⁺ + k⁻)(k⁺ + k⁻ + 2k₀)} \] \hspace{1cm} (3)

From luminescence decay analysis one finds the absolute value of E_q, but not its sign. Which Ln enantiomer is in excess in the excited state can be found from a CPL experiment. The magnitude of the CPL is usually expressed as the dissymmetry factor g_{lum} (14):

\[ g_{lum} = 2(I_L - I_R)/(I_L + I_R) \] \hspace{1cm} (4)

where I_L (I_R) denotes the intensity of left (right) circularly polarized emission light. Given the average quenching rate constant \( k_q \) (\( = k_0 (k_0 + k_\pm) \)), k₀ and the degree of circular polarization of the pure Λ-Ln enantiomer, g_{lum} the enantioselectivity E_q can be calculated from the result of a CPL measurement under continuous-wave excitation (g_{lum}) [14]:

\[ E_q = \frac{g_{lum}(\eta) k_\pm [Q] + k_0}{g_{lum}(\eta) k_\pm [Q]} \] \hspace{1cm} (5)

Results and discussion

Mitochondrial cytc

The lower part of Fig. 1 shows the pH dependence of the decay constants k⁺ and k⁻ for the quenching of Eu(DPA)₃⁻ and Tb(DPA)₃⁻ luminescence by horse heart ferricytochrome c. The ionic strength I of the sample solutions was adjusted to 22 mM by addition of NaCl because the quenching rates strongly depend on I [1]. It appears that the value of k₀ is not affected by pH in the range studied (Fig. 1), indicating the structural integrity of the Ln(DPA)₃⁻ species under these conditions (direct coordination of a water molecule to the Ln³⁺ ion, replacing one or more of the nine coordinated atoms of the three DPA ligands, leads to a drastic decrease of luminescence lifetime [17]). Looking first at the data pertaining to Eu, the decay constants show a strong reduction upon increasing pH. For pH > 10 the constants in the presence and absence of protein are almost identical: at these pH values the protein is an inefficient quencher of Eu luminescence.

We first discuss the pH dependence of the quenching in terms of a simple model in which the capability of the protein to quench is controlled by the protonation state of a single residue (with dissociation constant K_q). The protonated form HQ quenches with a rate constant \( k_q^p \) while the deprotonated form Q⁻ is assumed to be inactive as quencher (\( k_q^p = 0 \)). In this model the decay constants can, in analogy to Eq. 2, be expressed as the sum of k₀ and the product of the quenching rate constant of HQ and its concentration; see Eq. 7. \([Q]_0\) is the total concentration of the quencher.

\[ k_q^p (\text{pH}) = \frac{[Q]_0}{1 + 10^{\text{pH}-\text{pK}_q}} + k_0 \] \hspace{1cm} (7)

The solid lines in the lower part of Fig. 1 are fits of Eq. 7 to the experimental data points with \( k_q^p \) and pK_q as adjustable parameters. Data for the Δ and Λ enantiomers were fitted independently of each other and the results are listed in Table 1. It appears that the simple
model provides a satisfactory description of the dependence of the decay constants on pH.

For the quenching of $\Delta$-Eu* and A-Eu*, almost the same pK_a value (9.1 and 9.2, cf. Table 1) is found and thus only a very small pH dependence of the enantioselectivity; see the upper part of Fig. 1. As can be seen, the enantioselectivities from the $g_{cw}$ measurements agree with those from luminescence decay experiments.

In the model the enantioselectivity is predicted to be completely independent of pH for there is one quencher only and $E_q$ is an intrinsic property of the quencher, independent of its concentration. Since the model comprises only a single titratable residue on the protein influencing the quenching, one expects pK_a values only a single titratable residue on the protein independent of its concentration. Since the model contains a small (\(\sim 20\%\)) dependence of $E_q$ on pH and a small difference between the pK_a values, the simple model describes the observed phenomena fairly well.

Interestingly, the pK_a values found from the quenching are very similar to that determined for the alkaline transition of cytc from absorption measurements, suggesting that the alkaline conformer of cytc is a very inefficient quencher of Eu* luminescence. Because of this inefficiency we will neglect the presence of multiple conformers of the protein at high pH [4, 9]. The quenching results do not indicate the occurrence of other pH dependent changes of the protein, taking place at lower H^+ concentration than the alkaline transition.

For Tb a larger dissimilarity of the pK_a values for the two diastereomeric reactions is found, which is accompanied by a significant reduction of $E_q$ at higher pH, more clearly at variance with the portent of the model than in the Eu case.\(^2\) The data for quenching of Tb luminescence are shown in the right panel of Fig. 1. As can be seen, the results obtained with Ches buffer are slightly different from those obtained with ethanolamine buffer; the former were not included in the fit of Eq. 7. The resulting pK_a values, which are somewhat higher than for Eu, are listed in Table 1.

We have reported earlier that at pH = 7 the value of $E_q$ for cytc with Tb (+0.28) differs from that for Eu (+0.48) [1]. This discrepancy is intriguing because Tb(DPA)\(^{3-}\) and Eu(DPA)\(^{3-}\) (and the excited species) are isostructural, or nearly so [18–21], so that the binding of $\sigma$-Eu* and $\sigma$-Tb* to the protein is expected to be equal (i.e. identical binding site and association constant). The present study confirms the discrepant $E_q$ values for Eu and Tb (cf. Fig. 1) and moreover it reveals that the pH dependence of $E_q$ is different for the two lanthanide chelates.

Previously we have ascribed the discrepant $E_q$ values of Tb and Eu with cytc to different transition state geometries for the energy transfer from Tb* and Eu* [1]. Although it is conceivable that the changes in $E_q$ with pH may be explained by this general model, the pH dependencies lead us to propose tentatively a more detailed model: suppose there is a second binding site. For Tb*, this binding site is a quenching site as well, but not for Eu*. Such a situation can arise because the electronic interactions responsible for the Ln*→heme energy transfer rate $k_{ex}$ may have different distance and/or orientational dependencies for Eu and Tb. We propose that both sites have a neighbouring protonated amino acid residue and that when the residue is deprotonated, the site no longer contributes to the quenching owing to a reduced binding affinity for the negatively charged Ln*. The lower value of $E_q$ for Tb(DPA)\(^{3-}\) and its different pH dependence can be accommodated in this model if the second site loses its activity at higher pH than the first and if it has a lower intrinsic enantioselectivity than site 1 ($E_q<0.48$).

\[ E_q(pH) = \frac{[Q]_{0}}{K^0 + [Q]_{0}} + \frac{[Q]_{0}}{1-10^{pK_a \cdot \Delta pH}} + \frac{[Q]_{0}}{1-10^{pK_a \cdot \Delta pH}} + k_0 \]  

To account for the observed quenching rates at high pH we must have $10^{pK_a \cdot \Delta pH} < 10^{\alpha} k^0_q$. Inserting Eq. 8 in Eq. 3 we obtain:

\[ E_q(pH) = \frac{1}{1+10^{pK_a \cdot \Delta pH}} + \frac{[Q]_{0}}{1+10^{\alpha \cdot \Delta pH}} + k_0 \]  

with

\[ \alpha = \frac{k_{sy}}{k_{av}} \]  

This formula shows that also the change in $E_q$ is given by a simple titration curve but the apparent pK_a associated with the change in $E_q$ is shifted by $10\log(c)$ pH units, with c defined by Eq. 10. Thus if the alkaline conformer quenches with a different enantioselectivity, a change in $E_q$ is expected with apparent pK_a > pK_a + 1. The experimentally observed pH dependence of $E_q$ yields, however, the same pK_a as for the quenching rates. Thus also Eq. 9 is too restrictive to account for the observed pH dependence of the enantioselectivity.

\[ \begin{array}{cccc}
\text{Table 1} & \text{Fit parameters describing the pH dependence of the decay constants} \\
& pK_a^2 & pK_a^4 & 10^{-8} \times 10^{2} k_q^4 \left( M^{-1} s^{-1} \right) & 10^{-8} \times 10^{2} k_q^4 \left( M^{-1} s^{-1} \right) \\
\text{Tb(DPA)}^{3-} & \text{cytc} & 9.3 & 9.5 & 2.1 & 1.2 \\
& \text{wt cytc-550} & 9.7 & 9.8 & 2.4 & 1.9 \\
& K14E & 9.7 & 9.8 & 0.77 & 0.54 \\
& K99E & 9.8 & 9.8 & 0.48 & 0.44 \\
\text{Eu(DPA)}^{3-} & \text{cytc} & 9.1 & 9.2 & 1.2 & 0.42 \\
& \text{wt cytc-550} & 9.8 & 9.8 & 0.85 & 0.58 \\
\end{array} \]

\(^a\) Standard errors as obtained from the fit procedure: $\pm 0.03$ for data pertaining to cytc and $\pm 0.04$ for the cytc-550 proteins

\(^2\) If in the model also the deprotonated form of the quencher (Q) is allowed to quench the Ln* emission we may expect a change in $E_q$ as this quantity may be different for HO and Q^-.

We can take the quenching by Q\(^{-}\) into account, by replacing Eq. 7 by Eq. 8

\[ k^0_q(pH) = \frac{[Q]_{0}}{1-10^{pK_a \cdot \Delta pH}} + \frac{[Q]_{0}}{1-10^{pK_a \cdot \Delta pH}} + k_0 \]  

With

\[ \frac{1}{c} = \frac{1}{10^{pK_a \cdot \Delta pH}} \]  

This structural data for Eu(DPA)\(^{3-}\), Tb(DPA)\(^{3-}\) and Nd(DPA)\(^{3-}\) (as well as for several other lanthanide tris-DPA complexes) are available from the Cambridge Structural Database under entries SOPFYU, JEXWOY and SERYET, respectively, with reference to Shengzhi et al. [19].
In a simple description the drastically reduced ability of the alkaline conformer of cyt to accept energy from the lanthanide chelates may result (1) from the altered electronic structure of the heme group (which can affect $k_{at}$) or (2) from a lower binding constant ($K$) due to decreased electrostatic interaction in the Ln*-protein precursor complex. At first sight possibility 1 seems a viable explanation, for the disappearance of the 695 nm charge transfer (CT) transition upon increasing pH leads to a decreasing spectral overlap with the Eu $^5D_0$ transition (see Fig. 2), and thus expectedly to a lowering of $k_{at}$. However, the relatively strong Eu $^5F_2$ transition does not fit this picture. Moreover, with Tb – where a similar pH dependence of the quenching rates is observed – the spectral overlap with the CT band is virtually absent. Explanation 1 therefore being unlikely, we discuss (explanation 2).

Stelwagen and Cass [22] have shown that, at neutral pH, horse heart ferricyt can bind two Fe(CN)$_6^{3-}$ ions and that the ability to bind these anions is lost upon undergoing the alkaline transition. As our Ln chelates have the same charge as Fe(CN)$_6^{3-}$ we expect the (electrostatic) binding properties for both ions to be related, which implies that the protein’s reduced capability to quench Ln* may well be due to a reduced binding of the lanthanide chelate to the protein. Titrations of ferricyt [24] have shown that upon going from pH 7 to 10 (the isoelectric point of the protein), approximately seven protons are titrated. It is therefore likely that the quenching is influenced by deprotonation of several residues on the protein. If these deprotonations have similar $pK_a$ values (which seems indeed to be the case [24]) the overall effect may resemble a one-proton titration curve as in Eq. 7.

**Bacterial cyt-c-550**

In Fig. 3 the pH dependence of the decay constants of Tb(DPA)$_3^{3-}$ luminescence in the presence of wt, Lys14→Glu (K14E) and Lys99→Glu (K99E) cyt-c-550 are presented. The figure also shows the magnitude of $E_0$ as a function of pH. As can be seen, $E_0$ for K99E is markedly lower than for the other two proteins. For all proteins the chiral discrimination in the quenching is independent of pH in the region $7<\text{pH}<9$; for pH$>9$ the enantioselectivity shows a small reduction.

The diastereomeric quenching rates for wt cyt-c-550 start to decrease at higher pH than those pertaining to cyt: the associated $pK_a$ values, found by fitting Eq. 7 to the experimental data (see lines in Fig. 3), are higher. For wt cyt-c-550 with Eu, the same values for the dissociation constant are found as with the Tb chelate (see Table 1, data not shown). The apparent $pK_a$ values of the three cyt-c-550 proteins are equal within experimental error (9.8±0.1) and do not match the $pK_a$ values determined for the alkaline transition: 11.2, 11.1 and 10.8 for wt [7], K14E [8] and K99E [11], respectively.

Thus, there is no simple correlation between the change in 695 nm absorbance and the change in decay constant as a function of pH. It was found previously in our laboratory, using EPR spectroscopy, that for wt and K99E cyt, species with altered heme ligation are detectable only at pH$>10$ [11, 12].

Like in the case of cyt, spectral overlap considerations plead against an important role for $k_{at}$ in the explanation of the pH dependence of the quenching. Also for the cyt-c-550 proteins, we ascribe the change in the quenching rate to a smaller association constant $K$ of Ln$^*$ and the protein’s binding site, owing to reduced electrostatic attraction.

In contrast to the magnitudes of the quenching rate constants, the pH dependence of the rates is very similar for the K14E mutant and the wt protein. As can be calculated from Fig. 3, the quantity $k_{at}(\text{K14E})/k_q(\text{wt})$ remains approximately constant as the pH is raised. Therefore, the observed pH dependence of the wt protein cannot be ascribed solely to deprotonation of Lys14. A similar reasoning holds for the K99E mutant, so that also deprotonation of Lys99 alone cannot be held responsible for the pH dependence of the quenching rate of the wt protein.

Increasing the pH from 8 to 11 reduces the magnitude of the quenching rate constant of the three cyt-c-550 proteins by no less than a factor of 10; mutation of either Lys14 or Lys99 (which are the lysine residues closest to the exposed heme edge) to a glutamate pro-

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4 The binding sites for this ion have been identified by NMR spectroscopy [23]

5 In the pH region under study, the absorbance ($A$) at 695 nm is not constant. For the wt protein, the change of $A$(695 nm) is limited to 10% in the range 7.2<pH<10.5. Only at higher pH does $A$(695 nm) start to decrease rapidly. For K14E and K99E, changes in $A$ occur already at somewhat lower pH, but also here the large change of $A$(695 nm) takes place at higher pH
Fig. 3 pH dependence of the decay constants of Tb(DPA)$_3^{3-}$ luminescence by Fe(III) cytc-550 proteins. Left: wt cytc-550 (11 μM); middle: Lys 14→Glu cytc-550 (13 μM); right: Lys99→Glu cytc-550 (13 μM). [Tb]=1 mM, ionic strength I=22 mM (kept constant). In the upper plots ($E_q$ vs. pH), open circles pertain to decay measurements and solid ones to $E_{	ext{norm}}$. 

vides a reduction with a factor of 3–4 only. This suggests that, for all three proteins, deprotonation of a single lysine residue cannot account for the observed pH dependence and that there are several residues on the protein (other than Lys14 and 99) with $pK_a \approx 9.8$ which contribute to the electrostatic binding energy and whose deprotonation influences the binding strength of the negatively charged Ln complex. Considering the structure of the protein, possible residues involved are Lys16, 53, 84, 97 and 103. In this view, the $pK_a$ of Lys14 and 99 can be $\sim 9.8$ or even higher if the effect of deprotonation of the other residues involved in the (electrostatic) binding of the Ln complex is large enough to reduce the binding constant by more than a factor of 10. It seems unlikely that the $pK_a$ of Lys14 and 99 is lower than 9.8 since in this case one would expect a significant reduction of the quenching rate for the wt protein at pH $\sim 9.8$ which should not occur for the mutants. 

As can be seen from Fig. 3, such an effect is not observed.

Thus for both cytc and cytc-550 the decrease in quenching rate is attributed to deprotonation of several lysine residues near the exposed heme edge. The fact that cytc-550 has more negative charges than cytc (located mainly on the protein surface opposite to the heme edge) and correspondingly a lower $pI$ value (4.6 vs. 10.0) may explain why the lysine residues near the heme deprotonate at higher pH for cytc-550 than for cytc.

In the pH range 7–8, all three proteins show a variation of the quenching rate with pH which is substantially larger than predicted by extrapolation of the fitted curve based on the model of Eq. 7, which predicts that the quenching rate should be virtually independent of pH in this region. This again illustrates the limitations of the one-proton transition model. In the pH range 5–9, histidine residues or heme propionates may be titrated, and deprotonation of these groups may influence the quenching rate. The constancy of $E_q$ in this pH region strongly suggests that the deprotonations do not occur at the quenching site itself.

In conclusion, the rates for energy transfer between electronically excited Ln(DPA)$_3^{3-}$ ions and cytc-550 show a sharp reduction when the pH is raised, indicating a possibly localized change in the structure and/or charge state of the protein near the heme group. In this sense, these experiments provide a new tool to probe the active site of heme proteins. As discussed, the observed effects can be explained in terms of a lowering of the positive electrostatic potential around the exposed heme edge. Measurement of the enantioselectivity provides additional information; for instance, the constancy of $E_q$ in the pH interval 7–9.5 indicates the absence of major conformational transitions.

The pH-induced change of the enantioselective quenching by horse heart cytc coincides with the alkaline transition of the protein. The inefficiency of the quenching of excited Ln(DPA)$_3^{3-}$ by the alkaline conformer(s), compared to the species present at neutral pH, can most likely be explained in terms of a reduced binding constant of the lanthanide chelate to the protein. The altered electronic structure of the heme moiety of the alkaline conformer is probably less important. Variation of the enantioselectivity with pH is observed for Tb but not for Eu. We have tentatively interpreted this interesting observation in terms of an additional quenching site on the protein available to Tb but not to Eu. Using the luminescence quenching technique, competitive binding studies between lanthanide chelates and other anionic species may provide further information on anion binding sites of the protein.

In comparison with electron transfer studies (see, e.g. [25, 26]), energy transfer studies can yield complementary information. The rate of an electron transfer reaction between a negatively charged ion and, for example, cytc-550 will be influenced by pH-induced changes in the binding affinity and the midpoint potential of the protein. The energy transfer rate is of course

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6 For wt cytc-550 the midpoint potential varies considerably with pH [12]
not influenced by the changes in midpoint potential but rather by changes in the electronic structure of the heme chromophore and the binding affinity. In the case where it can be shown that the electronic structure changes only slightly with pH (like for cytc-550 in the interval 7＜pH＜10), energy transfer studies can be used to probe changes in the binding affinity and can provide information on the electrostatic potential near the active site of heme proteins.

References

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