pH Dependence of the enantioselective excited-state quencing of L,D-lanthanide(III) tris (pyridine-2,6-dicarboxylate) chelates by ferricytochrome c from horse heart and ferricytochrome c-550 from Paracoccus versutus

Meskers, S.C.J.; Ubbink, M.; Canters, G.W.; Dekkers, H.P.J.M.

JBIC (1998) 3:463–469

ORIGINAL ARTICLE

Stefan C. J. Meskers · Marcellus Ubbink
Gerard W. Canters · Harry P. J. M. Dekkers

pH dependence of the enantioselective excited-state quenching of Λ,Δ-Tb(III) and Λ,Δ-Eu(III)tris(pyridine-2,6-dicarboxylate) chelates by ferricytochrome *c* from horse heart and ferricytochrome *c*-550 from *Paracoccus versutus*

Received: 3 April 1998 / Accepted: 15 June 1998

Abstract The pH dependence of the dynamic quenching of the luminescence from Tb(III) and Eu(III) tris-(pyridine-2,6-dicarboxylate ≡ DPA) chelates by the title proteins is studied. For $Tb(DPA)_3^{3-}$ also the quenching by the Lys 14→Glu and Lys99→Glu mutants of cytochrome *c*-550 (cytc-550) is investigated. The rate constants for quenching of the electronically excited Λ and Δ 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 ± 0.1 for Eu and 9.4 ± 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>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 does not correlate with the alkaline transition, for which a pK_a of 11.2 has been reported by other workers. For all proteins, the reduction 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.

M. Ubbink · G. W. Canters · H. P. J. M. Dekkers
Leiden Institute of Chemistry, Gorlaeus Laboratories,
Leiden University, PO Box 9502, NL-2300 RA Leiden,
The Netherlands

S. C. J. Meskers (✉)
Laboratory of Macromolecular and Organic Chemistry,
Eindhoven University of Technology, PO Box 513,
NL-5600 MB Eindhoven, The Netherlands
e-mail: s.c.j.meskers@tue.nl
Tel.: +31-40-2473109, Fax: +31-40-2451036

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 μM) is added to an aqueous solution containing racemic (Λ,Δ)Ln(III) tris(pyridine-2,6-dicarboxylate ≡ DPA) chelate (~1 mM). We abbreviate $Ln(DPA)_3^{3-}$ 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

[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 excited lanthanide to the heme group of the protein. Owing to enantioselectivity in this process, the rate constants for quenching of Δ-Ln* and Λ-Ln* are different and the lifetimes of the two excited state populations are not equal any more. The overall reaction can be described by a pre-equilibrium step involving the formation of an encounter complex in which Ln* is bound to the protein (association constant K^{σ}, where σ denotes the sense of chirality of Ln, Λ or Δ), followed by the energy transfer step (rate constant k_{et}^{σ}).

A characteristic structural feature of the *c*-type cytochromes is that one edge of the heme group is exposed to the solvent. As the electronic interactions leading to energy transfer between the energy donating and accepting groups strongly decay with distance R ($\propto R^{-n}$, $n \geq 6$), energy transfer occurs predominantly from excited lanthanide chelates bound at the protein's surface close to the solvent exposed heme edge. Binding of the triply negative charged Ln species is electrostatically 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 residues 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. Moreover, mutation of Lys99 also affects the enantioselectivity in the quenching. These observations, together with theoretical considerations, have led us to propose a tentative 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 protons 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 enantioselective 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 conformational 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 potential compared to the native conformer and is no longer reduced by ascorbate, $Fe(CN)_6^{4-}$ or ferrocytochrome *c*1, 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 ($\varepsilon_{max} \approx 10^3$ cm^{-1} M^{-1}) disappears from the spectrum. The alkaline transition has been characterized by several other experimental techniques as well (see, e.g. [6]). Despite the extensive characterization, the underlying 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 lysine) as the sixth ligand of the central iron atom. The observation of several sets of heme methyl NMR signals at high pH indicates that several conformers of the protein exist and that more than one amino acid sidechain 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 proton characterized by a pK_a value of $\sim$8.5 and 9–9.4, respectively [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 homologous 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 enantioselectivity of the quenching of Eu- and $Tb(DPA)_3^{3-}$ 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 between changes in the quenching as a function of pH and the conformational transition of the protein. For Tb* luminescence the quenching by two site-specifically mutated cytc-550 proteins (K14E and K99E) will be investigated 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 *Escherichia coli* were performed as described.

Preparation of samples

The $Ln(DPA)_3^{3-}$ complexes were prepared as described earlier [14]. Protein concentrations were determined spectrophotometrically, using 8.4×10^3 M^{-1} cm^{-1} [15] and 10.3×10^3 M^{-1} cm^{-1} [7] as values for the extinction coefficients at 550 nm of oxidized cytc 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 $K_3Fe(CN)_6$. The results obtained with such samples were the same as measured in the absence of oxidant. $Fe(CN)_6^{3-}$ 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 $Ln(DPA)_3^{3-}$ complex, k^{Δ} and k^{Λ}, 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.

$$I(t) = I_0\{\exp(-k^{\Delta}t) + \exp(-k^{\Lambda}t)\} + (\text{dark counts}) \quad (1)$$

From the decay constants the quenching rate constants, k_q^{Δ} and k_q^{Λ}, can be calculated using Eq. 2:

$$k^{\sigma} = k_0 + k_q^{\sigma}[Q] \quad (2)$$

where k_0 is the decay constant in the absence of the quencher Q. The degree of enantioselectivity is defined as

$$E_q = (k_q^{\Delta} - k_q^{\Lambda})/(k_q^{\Delta} + k_q^{\Lambda}) = (k^{\Delta} - k^{\Lambda})/(k^{\Delta} + k^{\Lambda} - 2k_0) \quad (3)$$

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}:

$$g_{lum} = 2(I_L - I_R)/(I_L + I_R) \quad (4)$$

where I_L (I_R) denotes the intensity of left (right) circularly polarized emission light. Given the average quenching rate constant k_q^{av} [$= {}^1/_2(k_q^{\Delta} + k_q^{\Lambda})$], k_0 and the degree of circular polarization of the pure Λ-Ln enantiomer, g_{lum}^{Λ}, the enantioselectivity E_q can be calculated from the result of a CPL measurement under continuous-wave excitation (g_{lum}^{cw}) [14]:

$$E_q = \frac{g_{lum}^{cw}(\lambda)\,k_q^{av}[Q] + k_0}{g_{lum}^{\Lambda}(\lambda)\,k_q^{av}[Q]} \quad (5)$$

Results and discussion

Mitochondrial cytc

The lower part of Fig. 1 shows the pH dependence of the decay constants k^{Δ} and k^{Λ} for the quenching of $Eu(DPA)_3^{3-}$ and $Tb(DPA)_3^{3-}$ 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 k_0 is not affected by pH in the range studied (Fig. 1), indicating the structural integrity of the $Ln(DPA)_3^{3-}$ species under these conditions (direct coordination of a water molecule to the Ln^{3+} 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.

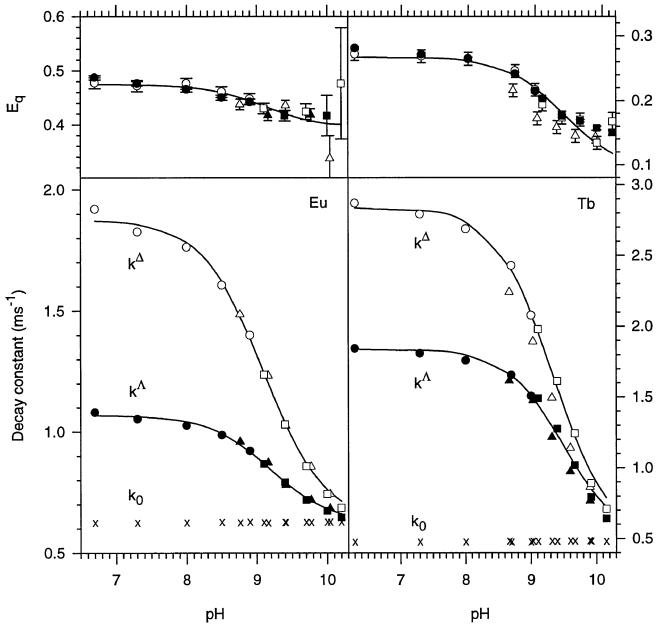

Fig. 1 pH dependence of the decay constants of $Ln(DPA)_3^{3-}$ luminescence in the absence (k_0) and in the presence (k^{Λ}, k^{Δ}) of ferricytc (*lower part*), and values of E_q (*upper part*). The shape of the symbols for the data points relates to the buffer (10 mM) used: Tris (○), ethanolamine (△), Ches (□). In the bottom plots, fits of Eq. 7 to the experimental data are shown as *solid lines*; fit parameters can be found in Table 1. The symbols used to represent E_q data have the following meaning: *open symbols* pertain to values from decay measurements; *solid symbols* to values from g_{lum}^{cw} measurements (see text). The error bars show the standard error of E_q. The solid line is not a fit to the E_q data points but is obtained by calculation of E_q from the fits to k^{Δ} and k^{Λ} shown in the lower part of the graph. Sample conditions: [Ln]=1 mM, [ferricytc]=11 μM, ionic strength I=22 mM (kept constant by addition of NaCl)

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_a). The protonated form HQ quenches with a rate constant ${}^{HQ}k_q^{\sigma}$ while the deprotonated form Q^- is assumed to be inactive as quencher (${}^{Q^-}k_q^{\sigma} = 0$). In this model the decay constants can, in analogy to Eq. 2, be expressed as the sum of k_0 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.

$$k^{\sigma}(\text{pH}) = {}^{HQ}k_q^{\sigma}\frac{[Q]_0}{1 + 10^{(\text{pH} - \text{p}K_a)}} + k_0 \quad (7)$$

The solid lines in the lower part of Fig. 1 are fits of Eq. 7 to the experimental data points with ${}^{HQ}k_q^{\sigma}$ and pK_a 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 Δ-Eu* and Λ-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_{lum}^{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 $pK_a^{\Delta} = pK_a^{\Lambda} = pK_a$. Although the experimental data show 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.

Table 1 Fit parameters describing the pH dependence of the decay constants

	pK_a^{Δ}	pK_a^{Λ}	$10^{-8} \times {}^{HQ}k_q^{\Delta}$ ($M^{-1}\,s^{-1}$)	$10^{-8} \times {}^{HQ}k_a^{\Lambda}$ ($M^{-1}\,s^{-1}$)
$Tb(DPA)_3^{3-}$				
cytc	9.3	9.5	2.1	1.2
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
$Eu(DPA)_3^{3-}$				
cytc	9.1	9.2	1.2	0.42
wt cytc-550	9.8	9.8	0.85	0.58

[a] Standard errors as obtained from the fit procedure: ± 0.03 for data pertaining to cytc and ± 0.04 for the cytc-500 proteins

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 $Tb(DPA)_3^{3-}$ and $Eu(DPA)_3^{3-}$ (and the excited species) are isostructural, or nearly so [18–21],[3] so that the binding of σ-Eu* and σ-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_{et} 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 $Tb(DPA)_3^{3-}$ 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 ($E_q < 0.48$).

[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 HQ and Q^-. We can take the quenching by Q^- into account, by replacing Eq. 7 by Eq. 8

$$k^{\sigma}(\mathrm{pH}) = {}^{HQ}k_q^{\sigma}\frac{[Q]_0}{1-10^{(\mathrm{pH}-pK_a)}} + {}^{Q^-}k_q^{\sigma}\frac{10^{(\mathrm{pH}-pK_a)}[Q]_0}{1-10^{(\mathrm{pH}-pK_a)}} + k_0 \qquad (8)$$

To account for the observed quenching rates at high pH we must have ${}^{HQ}k_q^{\sigma} > 10 \times {}^{Q^-}k_q^{\sigma}$. Inserting Eq. 8 in Eq. 3 we obtain:

$$E_q(\mathrm{pH}) = {}^{HQ}E_q\frac{1}{1+c10^{(\mathrm{pH}-pK_a)}} + {}^{Q^-}E_q\frac{c10^{(\mathrm{pH}-pK_a)}}{1+c10^{(\mathrm{pH}-pK_a)}} \qquad (9)$$

with

$$c = \frac{{}^{Q^-}k_q^{av}}{{}^{HQ}k_q^{av}} \qquad (10)$$

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

[3] Structural data for $Eu(DPA)_3^{3-}$, $Tb(DPA)_3^{3-}$ and $Nd(DPA)_3^{3-}$ (as well as for several other lanthanide tris-DPA complexes) are available from the Cambridge Structural Database under entries SOPFUY, JEXWOY and SERYET, respectively, with reference to Shengzhi et al. [19]

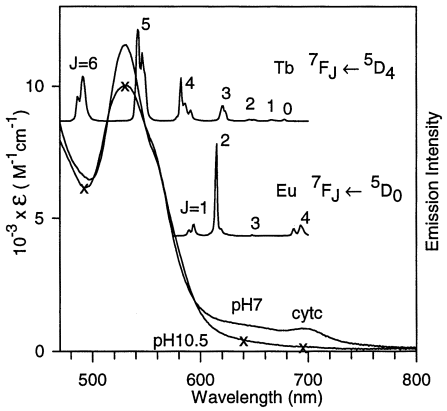

Fig. 2 Spectral overlap between the absorption spectrum of equine Fe(III) cytc at pH 7 (—) and pH 10.5 (–×–) and the emission from $Tb(DPA)_3^{3-}$ and $Eu(DPA)_3^{3-}$. The assignments of the emission bands are also given

In a simple description the drastically reduced ability of the alkaline conformer of cytc to accept energy from the lanthanide chelates may result (1) from the altered electronic structure of the heme group (which can affect k_{et}) 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 $^7F_4 \leftarrow {}^5D_0$ transition (see Fig. 2), and thus expectedly to a lowering of k_{et}. However, the relatively strong Eu $^7F_2 \leftarrow {}^5D_0$ 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 ferricytc can bind two $Fe(CN)_6^{3-}$ ions and that the ability to bind these anions is lost upon undergoing the alkaline transition.[4] 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 ferricytc [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.

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

Bacterial cytc-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) cytc-550 are presented. The figure also shows the magnitude of E_q as a function of pH. As can be seen, E_q 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 $7 < pH < 9$; for $pH > 9$ the enantioselectivity shows a small reduction.

The diastereomeric quenching rates for wt cytc-550 start to decrease at higher pH than those pertaining to cytc: the associated pK_a^σ values, found by fitting Eq. 7 to the experimental data (see lines in Fig. 3), are higher. For wt cytc-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 cytc-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.[5] It was found previously in our laboratory, using EPR spectroscopy, that for wt and K99E cytc, species with altered heme ligation are detectable only at $pH > 10$ [11, 12].

Like in the case of cytc, spectral overlap considerations plead against an important role for k_{et} in the explanation of the pH dependence of the quenching. Also for the cytc-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 $k_q^\sigma(\text{K14E})/k_q^\sigma(\text{wt})$ 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 cytc-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-

[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

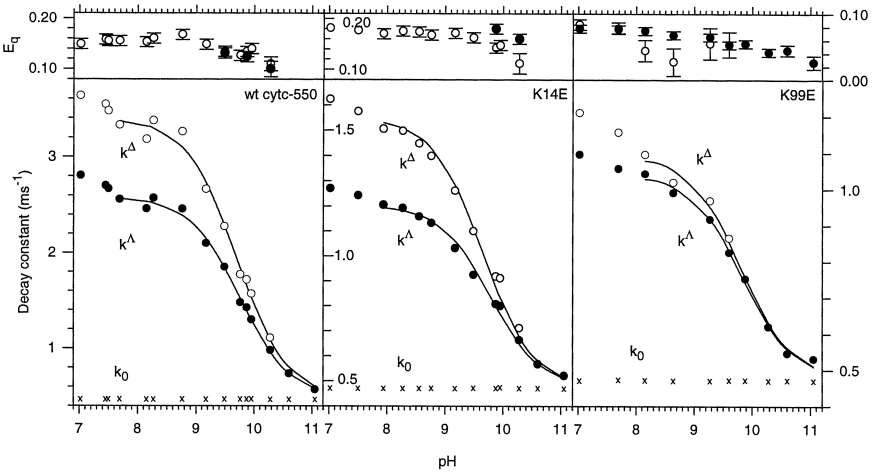

Fig. 3 pH dependence of the decay constants of $Tb(DPA)_3^{3-}$ luminescence by Fe(III) cytc-550 proteins. *Left:* wt cytc-550 (11 μM); *middle:* Lys 14→Glu cytc-550 (13 μM); *right:* Lys99→Glu cytc-550 (13 μM). [Tb] = 1 mM, ionic strength $I = 22$ mM (kept constant). In the *upper plots* (E_q vs. pH), *open circles* pertain to decay measurements and *solid ones* to g_{lum}^{cw}

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 ~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 < 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 stucture 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.[6] The energy transfer rate is of course

[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

1. Meskers SCJ, Ubbink M, Canters GW, Dekkers HPJM (1996) J Phys Chem 100:17957
2. Meskers SCJ, Dekkers HPJM (1998) Enantiomer (in press)
3. Katayama Y, Hiraishi A, Kuraishi H (1995) Microbiology 141:1469
4. Moore GW, Pettigrew GR (1990) Cytochromes c. Evolutionary, structural and physiochemical aspects. Springer, Berlin Heidelberg New York
5. Dickerson RE, Timkovich R (1975) In: Boyer PD (ed) The enzymes, vol 11. Academic Press, New York, chap 7
6. Wilson MT, Greenwood C (1996) In: Scott RA, Mauk AG (eds) Cytochrome c. A multidisciplinairy approach. University Science Books, Sausalito, Calif, 611–634
7. Lommen A, Ratsma A, Bijlsma N, Canters GW, Van Wielink JE, Frank J, Van Beeumen J (1990) Eur J Biochem 192:653
8. Ubbink M, Canters GW (1993) Biochemistry 32:13893
9. Ferrer JC, Guilletmette JG, Bogumil R, Inglis SC, Smith M, Mauk AG (1993) J Am Chem Soc 115:7507
10. Rosell FI, Mauk AG (1995) J Inorg Biochem 59:430
11. Ubbink M, Warmerdam GCM, Campos AP, Teixeira M, Canters GW (1994) FEBS Lett 351:100
12. Ubbink M, Campos AP, Teixeira M, Hunt NI, Hill HAO, Canters GW (1994) Biochemistry 33:10051
13. Ubbink M, Van Beeumen J, Canters GW (1992) J Bacteriol 174:3707
14. Rexwinkel RB, Meskers SCJ, Riehl JP, Dekkers HPJM (1992) J Phys Chem 96:1112
15. Van Gelder F, Slater EC (1962) Biochim Biophys Acta 58:593
16. Rexwinkel RB, Schakel P, Meskers SCJ, Dekkers HPJM (1993) Appl Spectrosc 47:73l
17. Horrocks W DeW Jr, Albin M (1984) Prog Inorg Chem 31:1
18. Albertsson J (1970, 1972) Acta Chem Scand 24:1213, 26:985, 26:1005, 26:1023
19. Shenghi H, Zhenchao D, Huizhen Z, Quiwang L (1989) Xiamen Dax Xuebao (Zir Kex) [J Xi Uni (Nat Sci)] 28:279, 28:514
20. Brayshaw PA, Harrowfield JM (1995) Acta Crystallogr C51:1799
21. Sherry AD, Geraldes CFGC (1989) In: Bünzli J-CG, Choppin GR (eds) Lanthanide probes in life, chemical and earth sciences. Elsevier, Amsterdam, 93–124
22. Stelwagen E, Cass RD (1975) J Biol Chem 250:2095
23. Arean CO, Moore GR, Williams G, Williams RJP (1988) Eur J Biochem 173:607
24. Shaw RW, Hartzell CR (1976) Biochemistry 15:1909
25. Bernauer K, Verano L (1996) Angew Chem Int Ed Engl 35:1716
26. Wishart JF, Hamada T, Sakaki S, Fujita E, Brunschwig B (1998) Abstr – Am Chem Soc 215:316