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The role of lysine 99 of *Thiobacillus versutus* cytochrome *c*-550 in the alkaline transition

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Abstract

The methionine ligand of the heme iron in ferricytochrome *c*-550 from *Thiobacillus versutus* is replaced by another residue at high pH. This transition is similar to the alkaline transition in mitochondrial cytochrome *c*. To investigate the possible role of lysine 99 in this process, this residue has been mutated to a glutamate. The mutation causes the apparent pK_a of the transition to decrease from 11.2 in wild type to 10.8 in Lys[99]Glu cytochrome *c*-550. This destabilization of the native form is ascribed to the absence of the hydrogen bond between the ε-amine group of Lys[99] and the carbonyl of Lys[54] in the mutant protein. The ^{1}H-NMR spectrum of Lys[99]Glu ferricytochrome *c*-550 at alkaline pH still shows resonance positions of the heme methyl peaks that are characteristic of the alkaline form. These results strongly suggest that Lys[99] does not act as a ligand in the high-pH form, contrary to the case of yeast iso-1-cytochrome *c*. Evidence has been presented that in the latter protein the homologous Lys[79] can act as a ligand in the alkaline form [1993, J. Am. Chem. Soc. 115, 7507–7508]. In the EPR spectrum of Lys[99]Glu cytochrome *c*-550 the species with Met-His coordination (g_z = 3.27) is replaced by two forms with g_z = 3.45 and 3.20 in the alkaline form (pH ≥ 10.6). At pH > 11 yet another form is observed with g-values 2.87, 2.18 and 1.60, tentatively identified as a species with a lysine-histidinate coordination of the heme iron.

Key words: Cytochrome *c*; Alkaline transition; EPR; Lysine; Heme coordination

1. Introduction

Cytochrome *c*-550 is part of a chain of redox proteins produced in the bacterium *Thiobacillus versutus* for the utilization of methylamine as carbon and nitrogen source [1]. It is a large type I cytochrome *c* [2–4] and shows a high homology with cytochrome *c*-550 from *Paracoccus denitrificans* [5]. The crystal structure of the latter protein shows that cytochrome *c*-550 is related to mitochondrial cytochrome *c* [6]. At neutral pH a His and a Met residue function as axial ligands of the heme iron in *T. versutus* cytochrome *c*-550, but at high pH the Met residue is replaced as a ligand in the ferric form of the protein, presumably by a lysine. This process is very similar to the alkaline transition in mitochondrial cytochrome *c* [7–10], although the pK_a is different. It is 11.2 in *T. versutus* cytochrome *c*-550 [11] and about 9 in mitochondrial cytochrome *c*, depending on the source of the protein [8,9]. The nature of this alkaline transition has been the subject of strong debate in the recent literature. The study of a cytochrome *c*-550 in which a well chosen point mutation has been applied, sheds new light on the origin of this transition. This is the subject of the present communication.

It is unknown which lysine replaces the methionine in *T. versutus* cytochrome *c*-550 at high pH. Fig. 1 shows that a number of lysines are close to the heme and are therefore likely candidates. Of these only Lys[14] can be discarded as a candidate because replacement by site-directed mutagenesis of Lys[14] has hardly any effect on the alkaline transition [11]. In mitochondrial cytochrome *c* two lysines appear to be able to replace the Met ligand; the alkaline form consists of (at least) two species [12], which probably differ in the lysine residue that acts as a ligand [13]. This inference is based on NMR measurements; the two species differ slightly in the resonance positions of the heme methyl groups. The chemical shifts of the heme methyl peaks as well as the concentration ratio of the two species depend on temperature [12]. Evidence has been presented that Lys[79] is one of the two lysines that act as ligands in yeast iso-1-cytochrome *c* [13]; mutagenesis of Lys[79] into an Ala residue resulted in the disappearance of one of the two species in the NMR spectrum of the alkaline cytochrome *c*. This result suggests that Lys[99] of cytochrome *c*-550, which is homologous to Lys[79] of mitochondrial cytochrome *c*, could be the ligand in alkaline cytochrome *c*-550. If so, removal of Lys[99] by site-directed mutagenesis should result in elimination of the alkaline transition. It is shown here, however, that such a mutant (Lys[99]Glu) still shows the transition, with a pK_a that is even lower than in wild type cytochrome *c*-550. This indicates that Lys[99] is not a ligand in the alkaline form. The evidence is reported here.

In addition the Lys[99]Glu protein appears to have the (unforseen) advantage that the pK_a of the alkaline transition has decreased with respect to the wild type protein. This enables a detailed analysis of the EPR features of the high pH species. In the wild type protein the complex appearance of the EPR spectrum precludes such a straightforward analysis.

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2. Materials and methods

2.1. Construction of mutation Lys^{99}Glu

Site-directed mutagenesis was performed as described [11]. The expression vector pMU19K99E is identical to pMU19 [5] except for the codon on position 99, which has been changed from AAG (lysine) to GAG (glutamate).

2.2. Purification of cytochrome c-550

Wild type cytochrome *c*-550 was isolated from *T. versutus* and *E. coli* and purified as described [5]. Lys^{99}Glu cytochrome *c*-550 was heterologously expressed in *E. coli*. Purification of the mutant was performed as in the case of heterologous wild type cytochrome *c*-550 [5] except that chromatography on S-Sepharose was done at pH 5.0 in 10 mM sodium acetate instead of at pH 6.0 in phosphate buffer.

2.3. Optical spectroscopy

Protein solutions of 60–70 μM in 10 mM sodium phosphate and 80 μM potassium ferricyanide (added to prevent autoreduction of cytochrome *c*-550 at high pH) were used to measure the absorbance at the α/β maximum and 696 nm as a function of pH. The pH was altered by addition of small aliquots of NaOH solution. Total volume was 2.2 ml. The sample holder was kept at 24°C.

2.4. NMR measurements

Protein samples for NMR were prepared by repeated dilution and concentration in D_2O in an Amicon stirred ultrafiltration cell with disc membranes from Millipore (PGLC025). Final concentration was 2 mM cytochrome *c*-550. The uncorrected pH* was adjusted to 11.6 by addition of small aliquods of NaOD-solution. Samples were flushed with argon to remove dioxygen. All measurements were performed on a 300 MHz Bruker WM300 spectrometer. Temperature settings were checked with a sample containing HDO and TMA in D_2O [14]. Free induction decays were stored in 16 K memory (spectral width 50 ppm). The HDO signal was suppressed by presaturation during 0.5 s. Exponential multiplication of the free induction decays was performed to increase the signal-to-noise ratio.

2.5. EPR measurements

X-band EPR was performed on a Bruker ESP380 spectrometer at 10 K. The experimental conditions were as follows. Microwave power: 2.4 mW, modulation amplitude: 0.90 mT, gain: 1×10^4, frequency: 9.422 GHz. A Cu(II) signal was observed in all samples both with and without protein. Spectra of buffer only were subtracted from spectra with buffer plus protein to correct for this artifact. CAPS (3-(cyclohexylamine)propanesulfonic acid, 50 mM) was used as a buffer for high-pH samples.

3. Results and discussion

3.1. Optical spectroscopy

To investigate the effect of mutation Lys^{99}Glu in *T. versutus* cytochrome *c*-550 on the alkaline transition the absorbance at 696 nm was measured as a function of pH. At high pH the 696 band disappears, like in wild type cytochrome *c*-550, indicating loss of Met coordination. Thus, the mutation does not prevent dissociation of the sulfur-iron bond. Like in wild type [11], the pH dependence of the 696 nm absorbance for Lys^{99}Glu cytochrome *c*-550 does not conform to a typical titration curve (Fig. 2A). It is clear, however, that the transition appears at a lower pH for the mutant than for the wild type protein. To characterise the pH dependency 'apparent pK_a' values have been determined as described elsewhere [11]. These amount to 10.8 for Lys^{99}Glu cytochrome *c*-550 and 11.2 for the wild type protein.

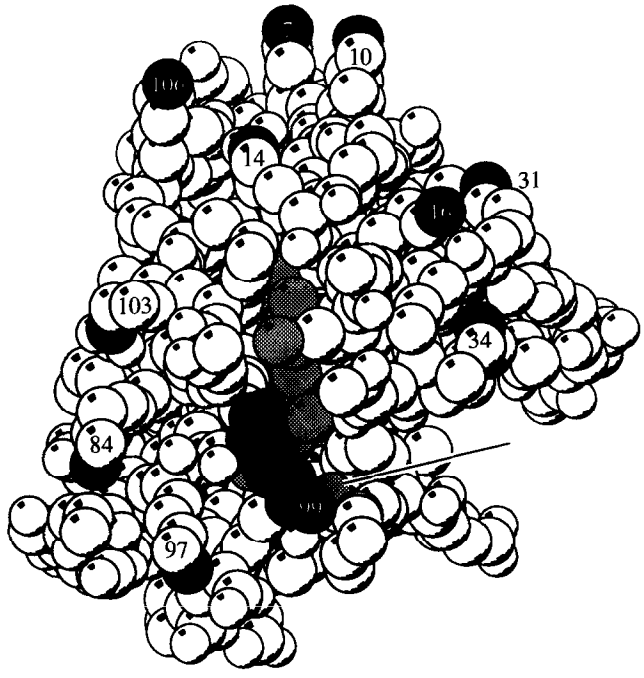

Fig. 1. Spacefilling model [20] of cytochrome *c*-550 from *T. versutus* based on the crystal structure data of cytochrome *c*-550 from *P. denitrificans* [6]. Black, N^{ζ} of lysine residues; dark grey, lysine 99; light grey, heme and carbonyl oxygen of lysine 54 (CO-54). The lysine side chains are numbered.

It has been proposed for mitochondrial cytochrome *c* that Lys^{79} is important in maintaining the native structure of the protein, because it forms a hydrogen bond with the carbonyl of residue 47 [15]. This bond is conserved in cytochrome *c*-550; the ε-amino group of Lys^{99} is close to the carbonyl of Lys^{54} (see Fig. 1). The absence of this hydrogen bond in Lys^{99}Glu cytochrome *c*-550 may therefore destablize the native form and lower the apparent pK_a of the alkaline transition.

The so-called α/β band (at 525 nm) of wild type ferricytochrome *c*-550 also changes at high pH. The extinction coefficient decreases by 15%, the band shape changes and the maximum shifts from 525 to 532 nm [11]. In the spectra of Lys^{99}Glu cytochrome *c*-550 the same phenomena are observed. The pH dependences of the intensities of the α/β and the 696 nm bands follow the same curve with the same apparent pK_a, in both wild type (not shown) and Lys^{99}Glu cytochrome *c*-550 (Fig. 2B). This suggests that the decrease of the α/β band intensity is a consequence of the alkaline transition. However, the shift of α/β band from 525 → 532 nm in Lys^{99}Glu cytochrome *c*-550 is not affected by the mutation; it shows the same pH dependence as the shift in the wild type protein, with an apparent pK_a of ca. 11.3 (Fig. 2C). Apparently, the shift of the α/β band is due to a pH dependent process other than the alkaline transition, as is discussed below.

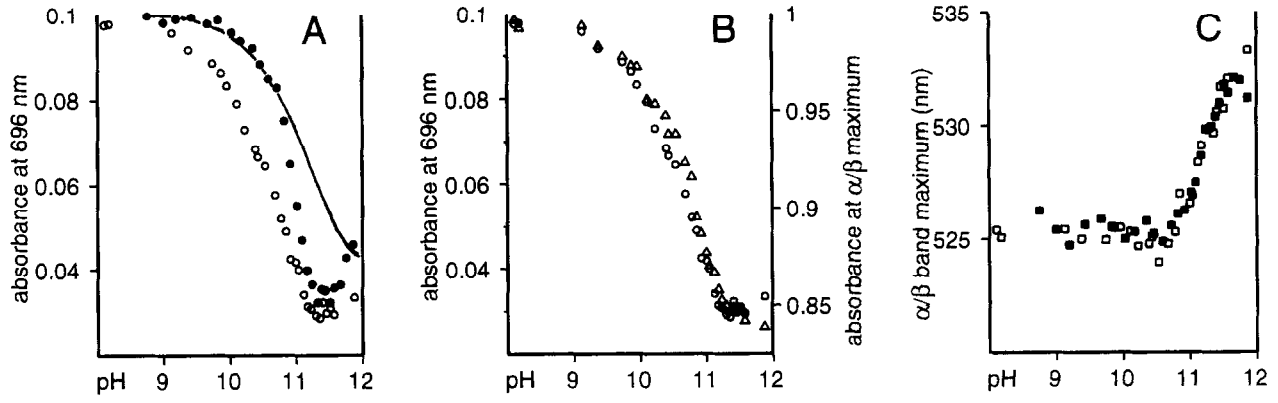

Fig. 2. The pH dependences of optical features of wild type (solid symbols) and Lys[99]Glu ferricytochrome *c*-550 (open symbols). Circles, absorption at 696 nm; triangles, absorption at the α/β-band maximum; squares, wavelength (nm) of the α/β-band maximum. (A) 696 nm absorption for wild type and Lys[99]Glu; the solid line represents a standard single proton titration curve with pK_a = 11.2. (B) 696 nm absorption and absorption at the α/β-maximum for Lys[99]Glu. (C) wavelength of the α/β-band maximum for wild type and Lys[99]Glu. Protein concentration was 60–70 μM in 10 mM sodium phosphate buffer.

3.2. NMR

The ^{1}H-NMR spectra of wild type and Lys[99]Glu ferricytochrome *c*-550 at pH* 11.6 are shown in Fig. 3. In the spectra both the native and alkaline form are present. This is best observed in the high frequency region. Two sets of heme proton peaks are observed; of these the resolved resonances of the heme methyls of the alkaline form are marked with asterisks. It is clear that the ratios of native and alkaline forms are different in the wild type and mutant spectra. This is in agreement with the lower apparent pK_a of the mutant as observed with optical spectroscopy. (Note that the apparent pK_a in D_2O is higher than in water as judged from the peak intensity ratios of native and alkaline form.) The mutation slightly affects the positions of the heme methyl peaks, both in the native and the alkaline form. Table 1 gives the chemical shifts of these peaks.

No indications are found in the NMR spectra for the existence two alkaline species as observed for mitochondrial cytochrome *c*; variation of the temperature did not resolve a second set of heme methyl peaks. It is concluded that *T. versutus* cytochrome *c*-550 shows one major alkaline form at ambient temperatures (298–313K). The presence of a minor species (<10%) cannot be excluded, however.

Table 1
Chemical shifts (ppm) of heme methyl peaks in ferric wild type and Lus[99]Glu cytochrome *c*-550 at pH* 11.6, T = 292K

Native form		Alkaline form	
Wild type	Lys[99]Glu	Wild type	Lys[99]Glu
12.40	12.74	9.58?	9.62
16.96	17.87	18.92	18.79
28.48	28.15	21.82	21.66
29.64	29.30	22.28	22.64

3.3. EPR

In Fig. 4 a pH titration of the X-band EPR spectrum of Lys[99]Glu cytochrome *c*-550 is presented. At neutral pH the mutant shows a species with g_z = 3.27 (Table 2, form I). At pH ≥ 10.6 two new species are observed with $g_z \approx 3.45$ and 3.2 (form II and III). Due to the proximity of the g-values for these species it was not possible to unambiguously deconvolute these spectra. At pH > 11.2 a species appears with g_z = 2.87 (form IV), which is the major form at pH > 12. The behaviour of wild type cytochrome *c*-550 at high pH is essentially the same as observed for the mutant protein except that the appearance of form II and III occurs at a somewhat higher pH.

This suggests that form II and/or III represent the alkaline form of the protein, with lysine-histidine coordi-

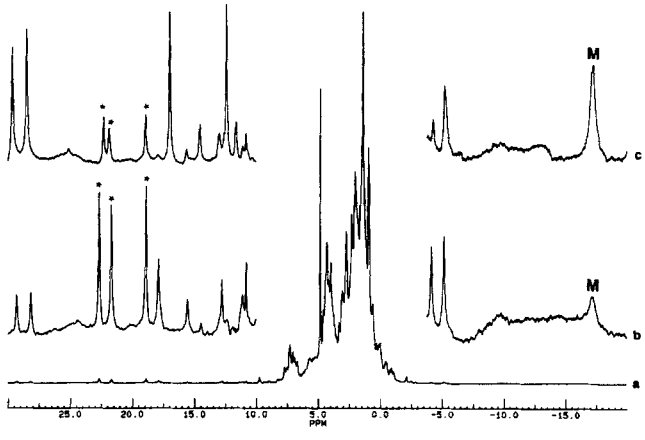

Fig. 3. 300 MHz ^{1}H-NMR spectrum of ferric wild type and Lys[99]Glu cytochrome *c*-550 at pH* 11.6. Protein concentration was 2 mM in D_2O; T = 292 K. (a) Full spectrum of Lys[99]Glu; (b) enlargement of resolved parts of the spectrum of Lys[99]Glu; (c) resolved parts of the wild type spectrum. The asterisks indicate heme methyl peaks of the alkaline form. M, peak assigned [4] to the methyl group of M100 (ligand in the native form).

nation of the heme iron. Similar spectra, with two EPR forms with g_z values of ca. 3.3 and 3.5, are also observed for alkaline mitochondrial cytochrome *c* [16] and for another mutant of *T. versutus* cytochrome *c*-550, Met[100]Lys (results to be published); both of these proteins are believed to have lysine-histidine coordination. The intensity ratio of the g_z = 3.3 and 3.5 species in the EPR spectra of the latter two proteins is strongly affected by the presence of buffers and co-solvents (e.g. glycerol) in the solution, while also the temperature may affect the relative concentration of the two species. Finally, the results of Ferrer et al. [13] on yeast Lys[79]Ala iso-1-cytochrome *c* indicate that no relation exists between the presence of two alkaline forms at ambient temperature (as observed with NMR) and the occurrence of two species in the EPR spectrum at 10K. The observation in the present case of one species of the cytochrome *c*-550 mutant in the NMR spectrum at room temperature in aqueous solution and of two species in the EPR spectrum of the protein at 10K are therefore not necessarily contradictory.

EPR form IV is tentatively assigned to a lysine-histidinate coordination of the heme iron, since the tetragonality (Δ/λ = 4.11 in the Blumberg and Peisach formalism [17]) of the species places it in a group of model heme compounds with histidinate coordination [18,19]. A more definite assignment may be obtained using magnetic circular dichroism in the near-infrared region on this high-pH form. It could be that the deprotonation that causes the appearance of form IV is also responsible for the shift of the α/β band in the optical spectrum at high pH, i.e. both spectroscopic features may represent the same process. The pK_a of this deprotonation is not affected by the Lys[99]Glu mutation.

3.4. Lysine 99 is not a ligand

The identity of the lysine ligand in the alkaline form is unknown. No indications exist that Lys[99] is the ligand in cytochrome *c*-550 at high pH. The Lys[99]Glu protein still shows the pH dependent changes in the optical and EPR spectra; also the heme methyl peaks characteristic of the alkaline form, are present in the NMR spectrum. In principle, it is still possible that Lys[99] is the ligand in the alkaline wt form and that another lysine residue takes over this role in the alkaline K99E cytochrome *c*-550.

Table 2
g-Values and crystal field parameters of EPR species of Lys[99]Glu cytochrome *c*-550

EPR forms	g_x	g_y	g_z	pH range	V/λ	Δ/λ	V/Δ	Proposed assignment
I	1.05[a]	2.05	3.27	<11.5	2.35	2.30	1.02	Met-His
II	n.d.[b]	n.d.[b]	≈3.45	10–11.9	–	–	–	Lys-His
III	n.d.[b]	n.d.[b]	≈3.2	10–11.9	–	–	–	
IV	1.60	2.18	2.87	>11.6	3.18	3.59	0.89	Lys-His⁻?

[a]Assuming $\Sigma g_i^2 = 16$; [b]n.d. = not detected.

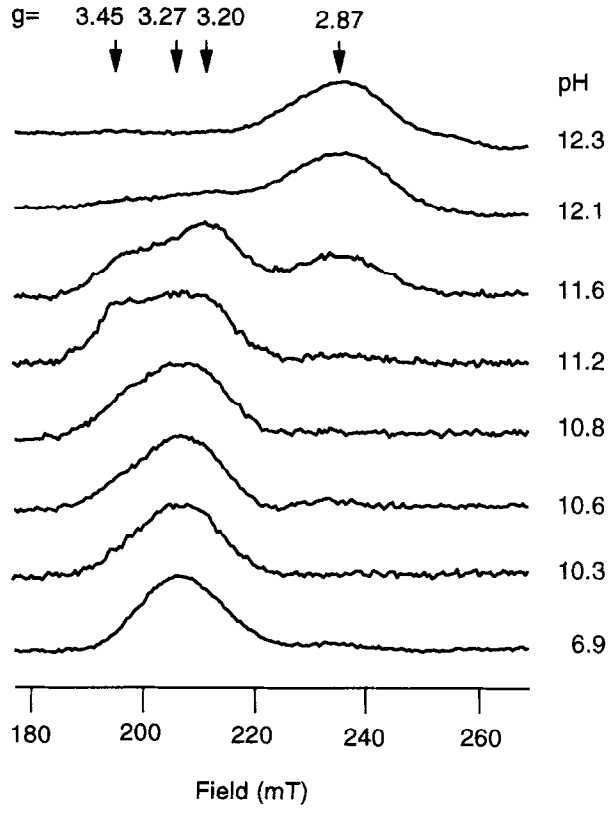

Fig. 4. Part of X-band EPR spectrum of Lys[99]Glu cytochrome *c*-550 (0.5 mM) as a function of pH. Microwave power: 2.4 mW; modulation amplitude: 0.90 mT; gain: 1 × 10[4]; frequency: 9.422 GHz; T = 10 K. The numbers indicate the g_z values of the peaks.

However, it may be expected that in that case the alkaline transition would be thermodynamically less favourable, resulting in a higher apparent pK_a, while what is found is that the mutation actually favours the alkaline tansition (lower apparent pK_a). Therefore the results seem to support the idea that Lys[99] does not act as a ligand in the alkaline form of cytochrome *c*-550. This is in contrast with the case of yeast iso-1-cytochrome *c*, in which the homologous Lys[79] is one of the ligands in the alkaline form [13]. From Fig. 1 Lys[84], Lys[97] and Lys[103] remain as likely candidates; of these Lys[84] appears to have the easiest access to the heme judged from the structure model. Lys[84] is conserved as K72 in mitochondrial cytochrome *c*, but in yeast iso-1-cytochrome *c* this lysine is trimethylated, which excludes this residue as a candidate [13], at least in the case of iso-1-cytochrome *c*. However, mitochondrial cytochrome *c* has another lysine very nearby, at position 73, which is not conserved in cytochrome *c*-550. Lys[103] of cytochrome *c*-550 is also conserved (as Lys[88]) but Lys[97] is not. Which of these acts as a ligand at alkaline pH could be decided by systematic site-directed mutagenesis.

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References

[1] Van Wielink, J.E., Frank Jnz., J. and Duine, J.A. (1989) in: PQQ and Quinoproteins (Jongejan, J.A. and Duine, J.A. eds.) pp. 269–278, Kluwer Academic Publishers, Dordrecht, The Netherlands.
[2] Ambler, R.P. (1980) in: From Cyclotrons to Cytochromes (Robinson, A.B. and Kaplan, N.O. eds.) pp. 263–279, Academic Press, London, UK.
[3] Pettigrew, G.W. and Moore, G.R. (1987) Cytochromes *c*, Biological Aspects, Springer-Verlag, Berlin, Germany.
[4] Lommen, A., Ratsma, A., Bijlsma, N., Canters, G.W., Van Wielink, J.E., Frank, J. and Van Beeumen, J. (1990) Eur. J. Biochem. 192, 653–661.
[5] Ubbink, M., Van Beeumen, J. and Canters, G.W. (1992) J. Bacteriol. 174, 3707–3714.
[6] Timkovich, R. and Dickerson, R.E. (1976) J. Biol. Chem. 251, 4033–4046.
[7] Brandt, K.G., Parks, P.C., Czerlinski, G.H. and Hess, G.P. (1966) J. Biol. Chem. 241, 4180–4185.
[8] Davis, L.A., Schejter, A. and Hess, G.P. (1974) J. Biol. Chem. 249, 2624–2632.
[9] Moore, G.R. and Pettigrew, G.W. (1990) Cytochromes *c*, Evolutionary, Structural and Physicochemical Aspects, Springer-Verlag, Berlin, Germany.
[10] Barker, P.D. and Mauk, A.G. (1992) J. Am. Chem. Soc. 114, 3619–3624.
[11] Ubbink, M. and Canters, G.W. (1993) Biochemistry 32, 13893–13901.
[12] Hong, X. and Dixon, D.W. (1989) FEBS Lett. 246, 105–108.
[13] Ferrer, J.C., Guillemette, J.G., Bogumil, R., Inglis, S.C., Smith, M. and Mauk, A.G. (1993) J. Am. Chem. Soc. 115, 7507–7508.
[14] Hartel, A.J., Lankhorst, P.P. and Altona, C. (1982) Eur. J. Biochem. 129, 343–357.
[15] Osheroff, N., Borden, D., Koppenol, W.H. and Margoliash, E. (1980) J. Biol. Chem. 255, 1689–1697.
[16] Gadsby, P.M.A., Peterson, J., Foote, N., Greenwood, C. and Thomson, A.J. (1987) Biochem. J. 246, 43–54.
[17] Blumberg, W.E. and Peisach, J. (1972) in: Probes of Structure and Function of Macromolecules and Membranes 2 (Chance, B. ed.) pp. 215–229, Academic Press, New York, NY.
[18] Gadsby, P.M.A. and Thomson, A.J. (1990) J. Am. Chem. Soc. 112, 5003–5011.
[19] Moore, G.R., Williams, R.J.P., Peterson, J., Thomson, A.J. and Mathews, F.S. (1985) Biochim. Biophys. Acta 829, 83–96.
[20] Kraulis, P.J. (1991) J. Appl. Crystallogr. 24, 946–950.