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Electronic Structure of the Two Copper Sites in Nitrite Reductase by 9 and 95 GHz EPR on Cavity Mutants

M. Fittipaldi[1,*], H. J. Wijma[2], M. P. Verbeet[2], G. W. Canters[2], and M. Huber[1]

[1] Department of Molecular Physics, Huygens Laboratories and [2] Metalloprotein Group, Gorlaeus Laboratories, Leiden University, Leiden, The Netherlands

Abstract. Electron paramagnetic resonance at 9 and 95 GHz on frozen solutions of the wild-type nitrite reductase (wt NiR) from *Alcaligenes faecalis* and on cavity mutants of its type 1 site has been performed to determine copper-hyperfine and g-tensor principal values of the type 1 and the type 2 copper sites. The mutants H145G, H145A, and M150G have a gap in the first coordination shell of the copper in the type 1 site. The reconstitution of the Cu site of the mutants by means of an external ligand such as imidazole or chloride was investigated. Information on the electronic structure of the type 1 site was obtained. Indications were found that the position of the histidine 145 in the native protein is not constrained by the protein environment but reflects the equilibrium position of this ligand with respect to Cu(II). Furthermore, changes in the electronic structure at the type 2 site induced by the modification of the type 1 site were detected, providing evidence for interaction between the two copper sites of the enzyme.

1 Introduction

Modern electron paramagnetic resonance (EPR) spectroscopy has proven a versatile tool to learn about the spatial and electronic structure of the active sites of proteins. The results of such investigations aid the understanding of the mechanism of protein function. The present study deals with active sites containing metal ions. To determine how the ligand environment affects the electronic structure of the metal site, cavity mutations were introduced. Such mutations replace an amino acid that forms a ligand of the metal with an amino acid that is less bulky, thus creating a gap (cavity) in the normally spacially dense interior of the protein. Thus, the effect of the absence of the ligand, or, by adding small molecules that can act as ligands to the metal ions, the effect of a change in the ligand can be investigated.

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* Present address: Department of Chemistry, University of Florence, Florence, Italy
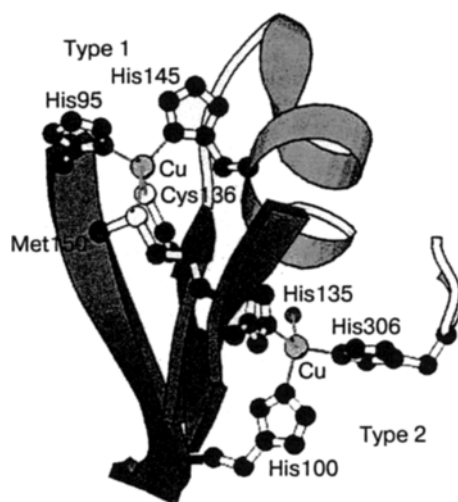
Fig. 1. Structure of the type 1 and type 2 copper sites of nitrite reductase. The copper in the type 1 site is bound to the two N_δ's of His95 and His145, the S_δ of Met150, and the S_γ of Cys136. The copper in the type 2 site is bound to three N_ϵ's of His135, His100, and His306. The two amino acid residues C136 and H135 link the type 1 to the type 2 site via the protein backbone.

The protein investigated, a copper-containing nitrite reductase (NiR), converts nitrite (NO_2^-) into nitric oxide (NO) and water. To perform this task, NiR uses two copper sites (Fig. 1): a type 1 site to acquire reducing equivalents in the form of electrons from redox-partner proteins, like pseudo-azurin in the case of *Alcaligenes faecalis* NiR [1, 2], and a type 2 site to catalyze the enzymatic conversion [3–5]. The 13-bond long covalent connection between the two copper centers provides for sufficient electronic coupling to guarantee a rate of electron transfer from the type 1 to the type 2 center that is compatible with physiological needs.

We have investigated cavity mutants at the type 1 copper site of NiR of *A. faecalis*. In the mutant H145G, the equatorial histidine at position 145 is replaced by a glycine and in H145A by an alanine, and in the mutant M150G, the axial ligand methionine is replaced by a glycine. For the mutants H145A and H145G, also reconstitution of the gap with imidazole and chloride, H145A(Im), H145G(Im), and H145A(Cl^-) was investigated. These modifications provide a means to study the function and the spectroscopic properties of the type 1 site in the catalytic cycle and to establish the importance of the ligands in the reaction of the enzyme [6, 7].

The relative ease by which the electronic structure of the type 1 site can be modified in these mutants also enables us to study the changes in the electronic structure of the type 2 site induced by a modification at the type 1 site, i.e., the investigation of the electronic coupling between the two copper sites of the enzyme.

We have performed continuous-wave (cw) EPR spectroscopy at 9 and 95 GHz on frozen solutions of the wild-type nitrite reductase (wt NiR), on the cavity mu-

tants and on their reconstituted counterparts. From EPR, information on the electronic structure of the type 1 site is obtained. Furthermore, evidence for changes in electronic structure at the type 2 site owing to the modification of the type 1 site is presented: For the various ligands that reconstitute the type 1 site, changes in the protein 1.25 nm away from the site of the modification are detected for this enzyme.

2 Materials and Methods

Preparation, protein isolation and reconstitution procedures of NiR, H145A, and H145G have been described elsewhere [6]. Preparation and protein isolation procedures of the NiR mutant M150G are described in ref. 7.

The solutions used for the EPR experiments had a concentration of 0.15–0.70 mM of protein with 30–40% of glycerol, depending on the mutants. The cw EPR measurements on frozen solutions were performed on a 9 GHz Elexsys E 680 spectrometer (Bruker, Rheinstetten, Germany). The spectra were acquired at 40 K. The amplitude and frequency of the modulation were 0.5 mT and 100 kHz, respectively. The spectra were recorded using a power of 0.6 mW and the total measurement time was of 21 min per spectrum. The number of accumulations was 30.

The W-band cw EPR measurement on the frozen solution was performed on a 95 GHz Elexsys E 680 spectrometer (Bruker). The amplitude and frequency of the modulation were 1.7 mT and 100 kHz, respectively. The spectra were recorded in a single sweep.

The cw EPR spectra of the various enzymes were simulated with the Bruker program Simfonia (Bruker, Rheinstetten, Germany). The g_{zz} and A_{zz} parameters of the two copper sites are easily obtainable by simulations of the X-band spectra. This particularly applies to the type 2 copper site, for which the signal at g_{zz} is more separated from the contribution of the type 1 signal and from the type 2 signal of molecules that have their resonance around g_{xx} and g_{yy}. The increased resolution at 95 GHz allows to resolve the g-values of the two copper sites also in the perpendicular region of the EPR spectrum (g_{xx} and g_{yy}). The estimated error from the W-band spectra is ± 0.002 for g_{xx} and g_{yy}. For spectra containing contributions of both copper sites, some of the parameters of the X-band EPR spectra are dependent on each other, resulting in larger errors for the A_{zz} and g_{zz} of the type 1 site compared with those of the type 2 site. The error is ± 0.3 mT for A_{zz} and ± 0.002 for g_{zz} of the type 1 site, while it is ± 0.1 mT for A_{zz} and ± 0.001 for g_{zz} of the type 2 site.

3 Results

Figure 2 shows the cw EPR spectra of frozen solutions of NiR and of several mutants acquired at X-band. In Table 1, the EPR parameters for both copper sites are reported as obtained from simulations (see Sect. 2). As indicated in Fig. 2a,

the EPR spectrum of wt NiR is a superposition of the EPR signals of the type
1 and the type 2 copper site. In particular, the EPR signals of the two copper
sites around g_{zz} are well separated from each other, while the signals of g_{xx} and
g_{yy} overlap around 0.33 T. This overlap can be resolved by EPR at higher mag-
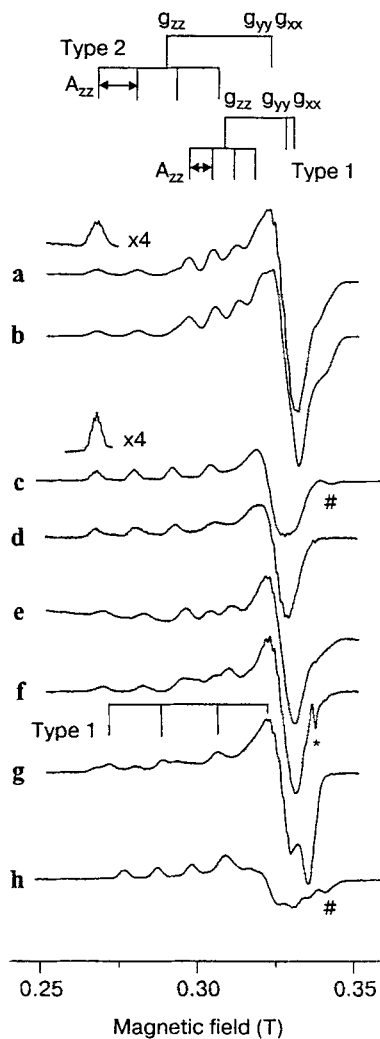netic field and frequency. Figure 3 shows the cw EPR spectrum of wt NiR ac-

Fig. 2. 9 GHz cw EPR spectra of frozen solutions of the enzymes: wt NiR (a), simulation of wt
NiR (b), H145A (c), H145G (d), H145G(Im) (e), H145A(Im) (f), H145A(Cl$^-$) (g), and H145A(NO$_2^-$)
(h). The insets are magnifications by a factor 4 of the feature at the lowest field of the spectra shown
in a and c. The spectra correspond to a microwave frequency of 9.477 GHz. Marked features: #,
artifacts of the spectrometer; *, small contamination.

Table 1. EPR parameters of wt NiR and NiR mutants.

Copper site and protein	A_{zz} (mT)	g_{zz}	g_{yy}	g_{xx}
Type 1 site				
NiR wt	7.5	2.1951[a]	2.055[a]	2.025[a]
H145G(Im)	7.7	2.195[b]		
H145A(Im)	5.7	2.195[b]		
H145A(Cl⁻)	17.4	2.275		
M150G	8.1	2.195[b]		
Type 2 site				
NiR wt	13.0	2.358	2.076[a]	2.076[a]
H145A	12.6	2.368	2.088	2.081
H145G	12.6	2.367		
H145A(Im)	13.0	2.343		
H145G(Im)	13.0	2.340		
H145A(Cl⁻)	13.0	2.358		
H145A(NO₂⁻)	10.9	2.315	2.130	2.045
M150G	13.0	2.358		

[a] From W-band experiments on frozen solutions, no absolute calibration. The g_{zz} value of the type 1 site was taken from the single-crystal EPR study of NiR of 2.1951 ± 0.0003 [14].

[b] The value of g_{zz} of the type 1 site in wt NiR has been used for the simulations. The estimated error from the W-band spectra is ± 0.002 for g_{xx} and g_{yy}. The error is ± 0.3 mT for A_{zz} and ± 0.002 for g_{zz} of the type 1 site, and ± 0.1 mT for A_{zz} and ± 0.001 for g_{zz} of the type 2 site (see Sect. 2).
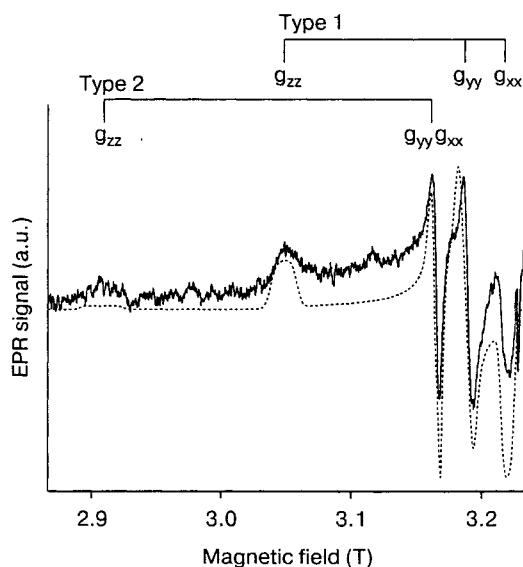

Fig. 3. 95 GHz cw EPR spectrum of a frozen solution of NiR (solid line) and its simulation (dotted line). The asterisk indicates a signal due to residual Mn^{2+} in the sample.

quired at 95 GHz (W-band). The higher magnetic field at W-band compared with X-band allowed to better resolve the g-anisotropy of the two centers, as well as to separate the contributions of the two copper sites. In particular, the 95 GHz EPR spectrum reveals the rhombicity of the type 1 site, while a potential rhombicity of the type 2 site could still be masked by the signal of the type 1 site. A simulation with an axial g-tensor for the type 2 site results in good agreement with the experimental spectrum. The EPR parameters g_{xx} and g_{yy} obtained from the W-band spectra are used in the simulation of the X-band EPR spectra. The information on the copper hyperfine coupling A_{zz} is lost at 95 GHz due to g-strain. Since g_{zz} and A_{zz} are the most relevant parameters for the changes in electronic structure, we focus on the X-band EPR results in the following.

In the X-band EPR spectra of the NiR mutants H145A and H145G, there is no signal from the type 1 copper site. Upon reconstitution of the site with imidazole a type 1 EPR signal reappears [6]. The EPR parameters of the type 1 site of H145G reconstituted with imidazole (H145G(Im)) are identical within experimental error to those of the wt NiR enzyme, while for H145A(Im) A_{zz} of the type 1 copper differs from wt NiR. Reconstitution with Cl$^-$ results in a different EPR signature of the type 1 site (Fig. 2) and substantially different EPR parameters compared with wt NiR.

In the mutant M150G, a type 1 signal is observed even without any external ligand added. The EPR parameters of this type 1 site are identical within experimental error to those of the type 1 site in wt NiR (Table 1).

The type 2 copper site of the mutants H145A and H145G is characterized by an A_{zz} value of 12.6 mT, which is smaller than the A_{zz} value of the type 2 site in wt NiR. The g_{zz} values of the type 2 signal of both mutants are larger than that of wt NiR. If the type 1 site in these mutants is reconstituted by imidazole (and Cl$^-$), the value of A_{zz} changes to 13.0 mT, which is equal to that of the type 2 site in wt NiR. The g_{zz} parameter of the type 2 site in H145A(Cl$^-$) is identical to that of wt NiR, whereas in H145A(Im) and H145G(Im) smaller g_{zz} values are observed. The mutant M150G has type 2 site parameters identical to those of wt NiR.

Comparison between the type 2 site signals in wt NiR, H145A(Im), and H145G(Im) on the one hand, and H145A and H145G on the other hand shows that in the latter case the width of the line at the lowest magnetic field in the EPR spectrum is reduced by 1.6 mT. Thus, in the absence of an oxidized type 1 site, the EPR signal of the type 2 site is narrower than if an oxidized type 1 copper is present. The binding of nitrite causes a significant change in the copper EPR parameters of the type 2 site: a reduction of both A_{zz} and g_{zz}, and even an increase in the rhombicity of the g-tensor.

4 Discussion

Variants of the NiR enzyme were investigated by cw EPR experiments at X-band and the EPR parameters of the two copper sites were obtained. The W-band EPR

spectrum resolves the rhombicity of the g-tensor of the type 1 site, whereas a rhombic contribution of the type 2 site becomes unlikely, but cannot be fully excluded. The preliminary conclusion from the W-band results is that the rhombicity of the type 2 site must be smaller than 0.01 ($g_{yy} - g_{xx}$), the value proposed in ref. 8. The rhombicity of copper sites has been related to the contribution of the d_{z^2} orbital to the SOMO, suggesting that the rhombicity can give information about the degree of admixture of the d_{z^2} orbital to the SOMO. Since the d_{z^2} orbital can provide coupling to the axially bound substrate, this admixture has mechanistic relevance.

The X-band EPR spectra of NiR reveal signals of the type 1 and the type 2 sites (Fig. 2), while only the signal of the type 2 site is observed in the EPR spectra of the H145G and H145A mutants. The copper content of these NiR mutants shows full occupancy of both sites, while the UV-Vis absorption bands typical of the oxidized type 1 site are absent, showing that the copper at the type 1 site is reduced to Cu(I) [6]. The reconstitution of the type 1 copper site is possible only in the presence of oxidants and external ligands (imidazole or chloride) as evidenced by the appearance of the band in the UV-Vis absorption spectrum typical of an oxidized type 1 site [6]. The EPR spectra also show the reappearance of a type 1 signal, i.e., reconstitution with imidazole results in an EPR signal of the copper type 1 site (Fig. 2). For H145G(Im), the EPR parameters of the reconstituted type 1 site are indistinguishable from those of the wt NiR, suggesting a similar coordination by the nitrogen of imidazole and that of histidine. The EPR parameters of the imidazole-reconstituted mutant H145A(Im), on the other hand, differ from those of the type 1 site in wt NiR. This suggests that in the larger cavity created by the Gly mutation at the type 1 site in H145G, the imidazole can adopt a position close to that of the imidazole ring of His 145 in the wt NiR. The additional methyl group of Ala in H145A makes the cavity smaller and seems to push the imidazole to a different position, as reflected in the different EPR parameters of the type 1 site in H145A(Im).

Reconstitution of the type 1 site in H145A by Cl$^-$, H145A(Cl$^-$), has been proven by X-ray crystallography and UV-Vis spectroscopy, revealing structural differences compared with the type 1 site in wt NiR [6]. The EPR spectra of H145A(Cl$^-$) consist of a superposition of two signals, one with EPR parameters close to the type 2 site in wt NiR and a second one with $g_{zz} = 2.275$ and $A_{zz} = 17.4$ mT. These observations allow to assign the second EPR signal to the reconstituted type 1 site, although its EPR parameters are quite different from those of the type 1 site in the native enzyme. This suggests the presence of an unusual type 1 site, in which blue color and high extinction coefficient characteristic of type 1 copper are combined with larger g_{zz} and A_{zz} values than commonly observed for such sites.

The observation that in the M150G mutant an intact type 1 site is present suggests that either an axial ligand is not required to cause the typical properties of the type 1 site, or that a ligand from the protein (sidechain or backbone) or from the buffer occupies the axial position. Crystallographic evidence supports the second possibility: In the mutant, the methionine at position 62 is found

at the position of the axial ligand, showing that a chemically identical ligand replaces M150. The similarity of the EPR parameters to those of the wt NiR shows that M62 is able to adopt a position structurally similar to M150 in wt NiR.

The EPR parameters of the type 2 copper site depend on the state of the type 1 copper site: For H145A and H145G (type 1 copper reduced to Cu(I)), the type 2 EPR parameters are identical within experimental uncertainty, but they differ from the EPR parameters of the type 2 site in wt NiR: A_{zz} is smaller and g_{zz} is larger than for wt NiR. If the type 1 sites of H145A and H145G are reconstituted with imidazole or chloride, a different set of EPR parameters is observed for the type 2 site. The A_{zz} values become close to those of the type 2 site in wt NiR, and the g_{zz} values become smaller (H145A(Im) and H145G(Im)) than those of wt NiR, or they become identical (H145A(Cl$^-$)) to wt NiR. No substantial differences between the structures of the type 2 sites in the mutants, the reconstituted mutants, and wt NiR have been found by X-ray crystallography [6].

The differences between the type 2 copper EPR parameters in reconstituted versus nonreconstituted mutants show that the type 2 site is sensitive to the state of the type 1 site of the enzyme, which is 1.25 nm away from the type 2 site, revealing that there is communication between the type 1 and type 2 sites. This could originate from: (a) a magnetic interaction, (b) an electrostatic interaction, (c) a small structural modification, or a combination of these factors. The magnetic dipole–dipole interaction between the two unpaired electrons at the two oxidized copper sites is of the order of 3 mT. Given the width of the line at the lowest magnetic field in the EPR spectrum (in the order of 5 mT) of the type 2 site, the oxidized copper at the type 1 site should cause a broadening of the lines of the type 2 signal. We attribute the larger line width of NiR variants with a type 1 copper in the oxidized state (wt NiR, H145A(Im), and H145G(Im)) to that magnetic interaction. The change in the A_{zz} and g_{zz} values of H145A and H145G with respect to NiR cannot be explained by the Cu(II)–Cu(II) magnetic-dipole interaction. Whether it is due to the small structural changes observed in the X-ray structure or a change in electronic properties of the type 2 site as a consequence of electrostatic interaction with the type 1 site remains to be determined. An effect of a change in the type 1 structure on the parameters of the type 2 site was previously reported [9] for another mutant of NiR in which the methionine axial ligand M150 of the type 1 copper was replaced by a glutamate (mutant M150E). In this case both g_{zz} and A_{zz} increase by approximately 3% relative to wt NiR, whereas in the H145 mutants g_{zz} increases and A_{zz} decreases. The more conservative modification at the type 1 site in the M150G mutant on the other hand has no effect on the copper type 2 EPR parameters. The M150E mutant differs from M150G in two respects: The glutamate introduces an extra charge and it was found that M150E preferentially binds Zn(II) at the type 2 site [10], suggesting that either one or both of these differences become transmitted to the type 2 site.

As expected, the replacement of a ligand at the type 2 site itself, such as replacement of water by nitrite, causes a more substantial change in the copper EPR parameters of the type 2 site, in particular, a strong increase in the rhom-

bicity of the g-tensor and a reduction in the A_{zz} parameter of the site. In addition, the hfc's of the nitrogens of the histidines decrease [11, 12], indicating that the spin density on copper and on the histidines diminishes. Spin density on the substrate itself is evidenced by the hfc of the nitrogen of nitrite determined by HYSCORE [12]. These findings suggest a shift of spin density from the ligands and the Cu(II) towards the substrate. The change in g-tensor parameters suggest a rearrangement of the copper d-orbitals, most likely a mixing in of d_{z^2} character into the wave function.

5 Summary and Outlook

In the present study, the effect of ligand replacement on the electronic structure of copper type 1 sites in NiR was investigated. From a spectroscopic point of view, the absence of the type 1 signal in the H145 mutants has been invaluable for studies of the electronic properties of the type 2 site, the catalytic site in this enzyme [12].

With respect to ligand replacement, we find that a ligand such as imidazole that is chemically similar to the (equatorial) His causes only a small change in the electronic structure of the site. The g_{zz} values are identical and the maximum variation in A_{zz} is 25%. Reconstitution with a ligand that changes the ligating atom and introduces an additional charge, such as Cl$^-$, drastically alters the EPR properties of the type 1 site, causing g_{zz} to increase by 4% and A_{zz} by 132%.

The result that the imidazole-reconstituted mutants H145A and H145G have different EPR parameters has further implications: Since the larger cavity of H145G results in EPR parameters for H145G(Im) that are indistinguishable from wt NiR, we conclude that the position of the imidazole ring of histidine in the native enzyme is not constrained by the protein backbone, but rather determined by the energetically most favorable position of the imidazole ring for the coordination to copper. This suggests that the ligands to the oxidized copper are in a relaxed position, i.e., not constrained by the protein environment.

Furthermore, evidence for the interaction of the type 1 site with the type 2 copper site in the enzyme was found. This advances our understanding of the interaction between the two copper sites in NiR that may be relevant for the function of this enzyme [8, 13, 12]. Whether electrostatic or structural effects dominate could be tested by introducing different metal ions at the type 1 site, such as, for example, Ag(I) or Zn(II).

The changes in the EPR parameters of the type 2 site upon substrate binding suggest a change in electronic structure to prepare the site for the chemical transformation. An admixture of the d_{z^2} orbital (increased rhombicity of the g-tensor) upon substrate binding provides for overlap of the wave function (SOMO) with the axially bound substrate. A smaller spin density at copper (smaller A_{zz}) and on the histidines (smaller nitrogen hyperfine couplings [11, 12]) and the spin density on the substrate [12] suggest that the SOMO coefficients shift towards

the substrate. Larger molecular-orbital coefficients of the SOMO on the substrate enable access of the electron needed for the reduction of nitrite and thus initiate the conversion to nitric oxide.

Obviously, a molecular interpretation of the changes in EPR parameters requires further experiments, such as the determination of the g-tensor in the copper type 2 site, as already performed for the type 1 site [14]. Such experiments are in progress.

Acknowledgments

References

1. Kakutani T., Watanabe H., Arima K., Beppu T.J.: Biochemistry **89**, 453–461 (1981)
2. Kakutani T., Watanabe H., Arima K., Beppu T.J.: Biochemistry **89**, 463–472 (1981)
3. Libby E., Averill B.A.: Biochem. Biophys. Res. Commun. **187**, 1529–1535 (1992)
4. Suzuki S., Kataoka K., Yamaguchi K., Inoue T., Kai Y.: Coord. Chem. Rev. **192**, 245–265 (1999)
5. Adman E.T., Murphy M.E.P. in: Handbook of Metalloproteins (Messerschmidt A., Huber R., Wieghardt K., Poulos T., eds.), pp. 1381–1390. Chichester: Wiley 2001.
6. Wijma H.J., Boulanger M.J., Molon A., Fittipaldi M., Huber M., Murphy M.E.P., Verbeet M.P., Canters G.W.: Biochemistry **42**, 4075–4083 (2003)
7. Wijma H.J., MacPherson I., Alexandre M., Diederix R.E.M., Canters G.W., Murphy M.E.P., Verbeet M.P.: J. Mol. Biol. **358**, 1081–1093 (2006)
8. Prudencio M., Eady R.R., Sawers G.: Biochem. J. **353**, 259–266 (2001)
9. Kukimoto M., Nishiyama M., Murphy M.E.P., Turley S., Adman E.T., Horinouchi S., Beppu T.: Biochemistry **33**, 5246–5252 (1994)
10. Murphy M.E.P., Turley S., Kukimoto M., Nishiyama M., Horinouchi S., Sasaki H., Tanokura M., Adman E.T.: Biochemistry **34**, 12107–12117 (1995)
11. Veselov A., Olesen K., Sienkiewicz A., Shapleigh J.P., Scholes C.P.: Biochemistry **37**, 6095–6105 (1998)
12. Fittipaldi M., Wijma H.J., Verbeet M.P., Canters G.W., Groenen E.J.J., Huber M.: Biochemistry **44**, 15193–15202 (2005)
13. Suzuki S., Deligeer, Yamaguchi K., Kataoka K., Kobayashi K., Tagawa S., Kohzuma T., Shidara S., Iwasaki H.: J. Biol. Inorg. Chem. **2**, 265–274 (1997)
14. van Gastel M., Boulanger M.J., Canters G.W., Huber M., Murphy M.E.P., Verbeet M.P., Groenen E.J.J.: J. Phys. Chem. B **105**, 2236–2243 (2001)

Authors' address: Martina Huber, Department of Molecular Physics, Huygens Laboratory, Leiden University, P.O. Box 9504, 2300 RA Leiden, The Netherlands
E-mail: mhuber@molphys.leidenuniv.nl.