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Published on Web 12/30/2006

Effect of the Methionine Ligand on the Reorganization Energy of the Type-1 Copper Site of Nitrite Reductase

Hein J. Wijma,[†,‡] Iain MacPherson,[§] Ole Farver,[||] Elitza I. Tocheva,[§] Israel Pecht,[⊥] Martin Ph. Verbeet,[†] Michael E. P. Murphy,[§] and Gerard W. Canters*[,†]

Contribution from the Leiden Institute of Chemistry, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands, Departments of Biochemistry and Molecular Biology and of Microbiology and Immunology, University of British Columbia, Vancouver, BC V6T 1Z3, Canada, Institute of Analytical Chemistry, The Danish University of Pharmaceutical Sciences, 2100 Copenhagen, Denmark, and The Weizmann Institute of Science, Rehovot 76100, Israel

Received July 5, 2006; E-mail: canters@chem.leidenuniv.nl

Abstract: Copper-containing nitrite reductase harbors a type-1 and a type-2 Cu site. The former acts as the electron acceptor site of the enzyme, and the latter is the site of catalytic action. The effect of the methionine ligand on the reorganization energy of the type-1 site was explored by studying the electron-transfer kinetics between NiR (wild type (wt) and the variants Met150Gly and Met150Thr) with Fe(II)EDTA and Fe(II)HEDTA. The mutations increased the reorganization energy by 0.3 eV (30 kJ mol^{-1}). A similar increase was found from pulse radiolysis experiments on the wt NIR and three variants (Met150Gly, Met150His, and Met150Thr). Binding of the nearby Met62 to the type-1 Cu site in Met150Gly (under influence of an allosteric effector) lowered the reorganization energy back to approximately the wt value. According to XRD data the structure of the reduced type-1 site in Met150Gly NiR in the presence of an allosteric effector is similar to that in the reduced wt NiR (solved to 1.85 Å), compatible with the similarity in reorganization energy.

Introduction

The rate of electron transfer between protein embedded redox centers is governed by the driving force $-\Delta G°$, the reorganization energy, λ, and the electronic coupling between the centers (A and B), H_{AB}.[1] Of these the $\Delta G°$ is often the easiest to determine; it simply equals the difference in midpoint potential of the two redox centers. The electronic coupling element, H_{AB}, is the most difficult. In principle, it can be calculated from a quantum mechanical treatment of the redox centers; in practice one has to take recourse to semiempirical or phenomenological treatments because the problem is of an unmanageable size, otherwise. The determination of the reorganization energy is of intermediate difficulty. According to Marcus theory it can be decomposed into contributions relating to individual self-exchange reactions, but even for self-exchange reactions the reorganization energies cannot be measured in a simple straightforward manner. One way is to measure the rate of electron transfer as a function of temperature, but the temperature range is often limited and it may be difficult to correct for temperature variations in the other parameters than the reorganization energy itself. Variation of the rate with driving force may be informative as well. Application of mutations and observing variations in the rate may give a clue to the magnitude of the reorganization energy, but again, making sure that other parameters are not affected by the mutations remains a challenge.

There is a need, therefore, for accurate and reliable values of reorganization energies for redox centers in proteins and a need, as well, for reliable techniques to measure them. Here we show how inorganic redox reagents can be used to obtain accurate values for the changes in reorganization energy when the redox site is modified by site-directed mutagenesis. The method is applied to a copper-containing enzyme. Cu containing proteins are interesting for an additional reason. After iron, copper is the most frequently used metal ion in nature to mediate electron transfer in biological systems. While synthetic copper complexes have a much higher λ than iron complexes (2.4 eV for Cu-phenanthroline[2] compared to 0.5−1.1 eV for various Fe complexes[3] (1 eV corresponds with 96.5 kJ mol^{-1})), the reorganization energies of biological copper-containing electron-transfer sites are similar to those of iron sites (0.6 eV for various c-type cytochromes,[4] 0.4 eV for a Cu_A site,[5] 0.6−0.8 eV for a

[†] Leiden University.
[‡] Current address: Duke University Medical Centre, Department of Biochemistry, Durham, North Carolina 27710.
[§] University of British Columbia.
[||] The Danish University of Pharmaceutical Sciences.
[⊥] The Weizmann Institute of Science.

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type-1 copper site[6−8]). It is of interest to try and investigate how the protein surroundings of a Cu site contribute to the reduction of the reorganization energy. One way to do that is to change the ligand sphere of the Cu site and to see how the reorganization energy changes.

Type-1 copper sites are found in small electron-transfer proteins (cupredoxins) and in enzymes, where they assist in internal electron transfer. In a type-1 site, three strictly conserved residues, i.e., two histidines and one cysteine, bind the copper. In addition, there are often one or two axial ligands like a methionine, a glutamine, or a backbone carbonyl oxygen.

To study variations in the reorganization energy connected with protein-mediated electron transfer we used the copper-containing nitrite reductase (NiR) from *Alcaligenes faecalis* S-6. Its physiological role is the dissimilatory reduction of nitrite ($NO_2^- + 2H^+ + e^- \rightleftharpoons NO + H_2O$).[9,10] NiR is a homotrimer, in which each subunit contains a type-1 copper site that accepts electrons from a physiological electron donor and transfers them to a catalytic type-2 copper site.[11−13] Replacing the axial methionine (Met150) by a glycine increases the midpoint potential (from 213 mV to 312 mV) and decreases the catalytic rate (from 416 s^{-1} to 133 s^{-1}).[14] Addition of allosteric effectors, like acetamide (AcM), brings the Met150Gly variant in a rearranged state where binding of Met62 at the original position of the Met150 restores catalytic activity and midpoint potential.[14]

Here we report on the effect of the removal or replacement of the axial methionine in Met150Gly, Met150His, and Met150Thr NiR and the subsequent binding of Met62 (in the case of Met150Gly) on the reorganization energy of the type-1 site by studying their reduction by synthetic electron donors (Met150Gly and Met150Thr) or radicals generated by pulse radiolysis (Met150Gly, Met150His, and Met150Thr). The results show that removal of the axial methionine (Met150) increases the reorganization energy by 0.3 eV (30 kJ mol^{-1}) and that binding of Met62 restores the reorganization energy to the level of the wt NiR consistent with information from XRD studies.

Materials and Methods

Electron Transfer and Reorganization Energy. Wt NiR and its variants were prepared as described.[14] The oxidized type-1 site has strong absorption bands around 600 nm (and around 460 nm for some variants) which are used to follow the reduction of this site. For the stopped-flow experiments an Applied Photophysics SX.18MV apparatus, equipped with oxygen-impermeable flow lines and set at a temperature of 25 °C, was used. High purity argon was used to deoxygenate solutions. Remaining traces of oxygen were removed by scrubbing the gas stream with a solution containing 0.5 mM methylviologen, 50 μM proflavin, 75 mM EDTA, and 50 mM of phosphate pH 6.0.[15] Butyl rubber and PEEK (Rheodyne) were used in the experimental setup to protect the gas stream and anaerobic solutions from oxygen. Fe(II)EDTA [Fe(II) ethylenediaminetetraacetate] and the Fe(II)HEDTA [Fe(II) *N*-(2-Hydroxyethyl)ethylenediamine-*N*,*N*′,*N*′-triacetate] solutions (always >10 times excess when reacted) were prepared at constant ionic strength (I = 0.2 M, kept constant by adjusting the sodium phosphate concentration, pH 7.0[16]). All reduction potentials are quoted with respect to the normal hydrogen electrode (NHE).

In pulse-radiolysis experiments, the NiR was reduced by 1-methylnicotinamide radicals as described elsewhere.[17] Since 1-methylnicotinamide (5 mM), *tert*-butanol (0.1 M), and MOPS (7 mM) are present in the reaction mixture for the pulse-radiolysis experiments, it was checked that these compounds do not compromise the experiments by binding to the cavity created by the Met150Gly mutation (results not shown).

The visible absorption of the type-1 site of NiR was used to study its reduction by ferrous EDTA in a stopped-flow experiment (eq 1, in which $T1^{OX};T2^{OX}$ is the NiR with both the type-1 and the type-2 site in the Cu(II) state, $T1^{RED};T2^{OX}$ is NiR with the type-1 site in the Cu(I) state, etc., $K_{eq} = [T1^{OX};T2^{RED}]/[T1^{RED};T2^{OX}] = k_2/k_{-2}$).

$$T1^{OX};T2^{OX} \xrightarrow{k_1'[\mathrm{Fe(II)EDTA}]} T1^{RED};T2^{OX} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} T1^{OX};T2^{RED} \xrightarrow{k_3'[\mathrm{Fe(II)EDTA}]} T1^{RED};T2^{RED} \quad (1)$$

Following the reaction sequence presented in eq 1, the absorbance $A(t)$ of the type-1 site (due to the species $T1^{OX};T2^{OX}$ and $T1^{OX};T2^{RED}$) is given by (see Supporting Information)

$$A(t) = A_0\left(e^{-k_1 t} + \left\{\frac{k_x}{k_3 - k_x}\right\}\{e^{-k_x t} - e^{-k_3 t}\}\right) \quad (2)$$

with A_0 the absorbance at $t = 0$, $k_1 = k_1' \times [\mathrm{Fe}]$, $k_3 = k_3' \times [\mathrm{Fe}]$, [Fe] being the concentration of reductant, $K_R = 1/K_{eq}$, and $k_x \equiv k_1/(K_R + 1)$. From the data analysis it will transpire (vide infra) that $k_1 = k_3$, which reduces eq 2 to

$$A(t) = A_0((1 - K_{eq})e^{-k_1 t} + K_{eq}e^{-k_x t}) \quad (3)$$

The measured rates were used to obtain values of $\Delta\lambda$ by employing eq 4 which was derived on the basis of Marcus theory[1] assuming that the only parameters which vary due to the mutations are driving force and reorganization energy (see Supporting Information):

$$\Delta\lambda = -8RT\ln\left(\frac{k_m'}{k_w'}\right) + 4F\Delta E \quad (4)$$

Here, k_m' and k_w' denote the k_1' values for wt and mutant NiR, respectively, $\Delta\lambda$ is the change in reorganization energy of the type-1

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site due to the mutation ($\Delta\lambda \equiv \lambda_m - \lambda_w$), F is the Faraday constant, R is the gas constant, T is the absolute temperature, and ΔE is the change in midpoint potential of the type-1 site due to the mutation ($\Delta E \equiv E_m - E_w$). The reorganization energies of the mutant type-1 sites were obtained from pulse-radiolysis data as described.[6,17]

Crystal Structure. Met150Gly crystals were grown at room temperature by the hanging drop vapor diffusion method, as described previously.[14] The mother liquor consisted of 6−10% PEG6000, 10 mM sodium acetate, pH 4.5, 2 mM zinc acetate, and 2 mM $CuSO_4$. Crystals were moved to the mother liquor without $CuSO_4$ for 30 min to remove excess copper, then to the copper-free mother liquor containing 1 M acetamide for 10−20 min, and then to Buffer A (copper-free mother liquor containing 1 M acetamide and 20 mM ascorbic acid) until they became colorless due to reduction. Last, crystals were transferred into Buffer A plus glycerol as a cryoprotectant. Wt NiR crystals were grown according to the same protocol with 0.1 M sodium acetate, pH 4.0. Reduced crystals were obtained as for the case for Met150Gly NiR (but without acetamide). Reduced crystals were looped into a cryostream (Oxford Cryo Systems) for diffraction studies using a MAR345 detector and Rigaku RU-300 X-ray generator and diffracted to better than a 1.9 Å resolution. Diffraction data were processed with DENZO.[18]

Reduced crystals contain the NiR trimer in the asymmetric unit of space group $P2_12_12_1$. For reduced Met150Gly-AcM, a 1.4 Å resolution structure of nitrite-soaked wt NiR[19] was used as the starting model for refinement after the removal of the Met150 side chain and nitrite. For reduced wt NiR, a 1.3 Å resolution structure of nitric oxide-soaked ascorbate-reduced wt NiR[19] was used as the starting model after the removal of nitric oxide. The structures were refined using REFMAC[20] with 5% of the data set aside for calculation of the free R-factor. The copper ligand geometry and positions of the copper atoms were not restrained throughout the refinement. In reduced Met150Gly-AcM, chain A begins with Thr5 and ends with Gly339 while chains B and C begin with Ala4 and end with Gly339. In reduced wt NiR, all chains begin with Ala4 and end with Gly339. Statistics of data processing and structure refinement are presented in Table 1.

Results

Reduction of NiR Followed by Stopped-Flow. To determine the reorganization energy of the modified type-1 sites we studied the reduction of wt, Met150Thr, and Met150Gly by Fe(II)EDTA and Fe(II)HEDTA while monitoring the absorption at 600 nm or 460 nm versus time. The midpoint potential of Met150His (104 ± 5 mV) is too close to that of the reductants (Fe(II)-EDTA: 120 mV; Fe(II)HEDTA: 47 mV) for reverse electron transfer to be neglected in the analysis, and reduction of Met150His was not studied, therefore. When wt NiR was reduced with either Fe(II)EDTA or Fe(II)HEDTA, traces were observed as depicted in Figure 1A. A double exponential fit yielded amplitudes of opposite sign, showing that $K_{eq} > 1$ (see eq 3 and Supporting Information). When fitted using eq 2 the values obtained for k_1 and k_3 were identical within experimental error and eq 3 was used throughout. Simulations of the absorbance of $T1^{OX};T2^{OX}$ and $T1^{OX};T2^{RED}$ versus time confirmed the buildup of a $T1^{OX};T2^{RED}$ intermediate (Figure 1A, inset).

For the Met150Thr and Met150Gly variants of NiR double exponentials with only positive amplitudes were observed (Figure 1B) meaning that $K_{eq} < 1$. The reduction of Met150Gly in the presence of 750 mM acetamide gave biphasic kinetics. For Met150Thr it was not possible to fit all parameters at once due to the low amplitude of the slower phase. Fixing $1/K_{eq}$ at 30 produced satisfactory fits (Figure 1B). Values of $1/K_{eq}$ of 15 or 60 were clearly too low or too high (not shown) and were therefore quoted as the error margins (Table 2). The dependence of the rates on reductant concentration was linear in all cases (Figure 2). From the known midpoint potentials of the type-1

Table 1. Crystallographic Data Collection and Refinement Statistics for Reduced M150G-Acetamide and Reduced wt AfNiR

structure	reduced M150G-AcM AfNiR	reduced native AfNiR
cell dimensions (Å)	$a = 61.0$	$a = 61.5$
	$b = 102.0$	$b = 102.4$
	$c = 146.4$	$c = 145.7$
resolution (Å)	1.90 (1.97−1.90)[a]	1.85 (1.92−1.85)
R-merge	0.071 (0.400)	0.116 (0.413)
$\{I\}/\{\sigma(I)\}$[b]	15.6 (2.49)	12.3 (4.7)
completeness (%)	94.7 (90.1)	91.5 (100)
unique reflections	68 966 (6488)	72 738 (7855)
working R-factor	0.175	0.150
free R-factor	0.220	0.180
rmsd bond length (Å)	0.014	0.010
overall B-factor (Å^2)	27.6	17.3
estimate of coordinate error[c]	0.182	0.150
ESU (maximum likelihood)[c]	0.104	0.072
water molecules	869	1106
PDB entry code	2B08	2FJS

[a] Values in parentheses are for the highest resolution shell. [b] $\{I\}/\{\sigma(I)\}$ is the average intensity divided by the average estimated error in intensity. [c] The coordinate error estimated by Cruickshank's DPI and the maximum likelihood ESU are as calculated by REFMAC.[20]

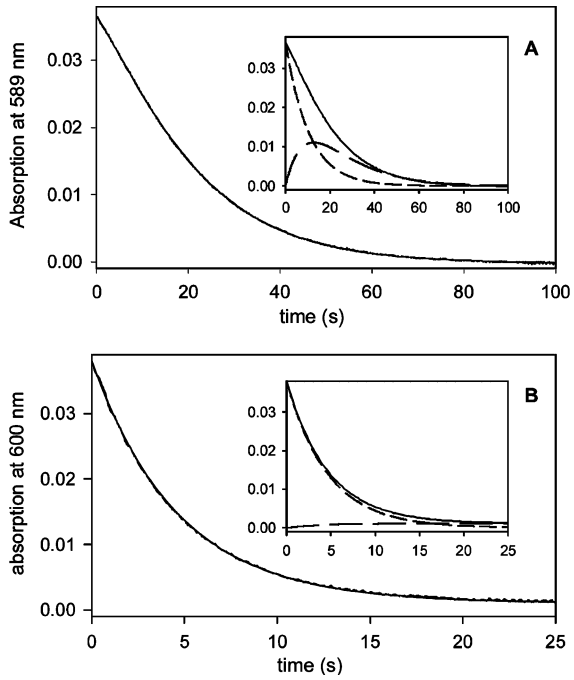

Figure 1. Reduction of NiR by Fe(II)EDTA. (A) Reduction of wt NiR (10 μM) by 2 mM Fe(II)HEDTA (pH 7.0, 298 K) at constant ionic strength (I = 0.2 M); actual data and fit to eq 3 depicted in the main window. A simulation of the absorbance of $T1^{OX}T2^{OX}$ (short dashes), $T1^{OX}T2^{RED}$ (long dashes), and their sum (continuous line) is shown in the inset; for details see text. The axes of the inset are identical to those of the main window. (B) the same for the reduction of NiR Met150Thr by 2 mM Fe(II)HEDTA.

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Table 2. Kinetic and Thermodynamic Constants Determined from the Reduction of NiR Variants with Inorganic Donors (pH 7.0, $T = 298$ K)

NiR variant	reductant	acetamide (mM)	k' ($M^{-1}\,s^{-1}$)	K_{eq}	E_M of the type-2 site[b] (mV)	$\Delta\lambda$ (eV)
WT	FeHEDTA	0	51.5 ± 0.2	1.5 ± 0.5	224 ± 14	0
WT	FeHEDTA	500	55.6 ± 0.6	1.7 ± 0.6	226 ± 14	-0.02 ± 0.04
WT	FeHEDTA	750	56.9 ± 0.4	1.8 ± 0.3	228 ± 11	-0.02 ± 0.04
Met150Thr	FeHEDTA	0	144.3 ± 1.3	0.033[c]	253 ± 23	0.30 ± 0.04
Met150Gly	FeHEDTA	0	83.2 ± 2.5	ND	ND	0.31 ± 0.05
Met150Gly	FeHEDTA	500	45.7 ± 0.6	0.13 ± 0.04	225 ± 12	<0.07
Met150Gly	FeHEDTA	750	51.6 ± 0.7	0.24 ± 0.03	234 ± 9	<0.05
WT[a]	FeEDTA	0	119 ± 3	2.5 ± 0.7	236 ± 12	0
Met150Gly	FeEDTA	0	185 ± 2	0.083 ± 0.031	251 ± 14	0.32 ± 0.03
Met150Gly	FeEDTA	500	98.7 ± 1.2	0.22 ± 0.04	239 ± 9	<0.09
Met150Gly	FeEDTA	750	97.9 ± 1.5	0.25 ± 0.02	235 ± 7	<0.09

[a] In the presence of 750 mM acetamide, traces identical to those without acetamide were obtained. [b] This was calculated with the known type-1 site midpoint potentials[14] E_M wt $= 213 \pm 5$ mV, E_M Met150Gly $= 312 \pm 5$ mV, E_M Met150Thr $= 340 \pm 5$ mV, E_M Met150Gly in the presence of 500 mM acetamide $= 278 \pm 5$ mV, E_M Met150Gly in the presence of 750 mM acetamide $= 271 \pm 5$ mV. [c] This value is in between 0.017 and 0.067. For experimental details see Materials and Methods. ND, not determined.

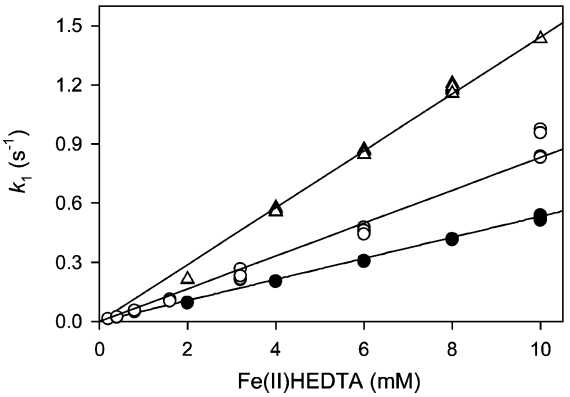

Figure 2. Rate of reduction of NiR versus reductant concentration. (△) Met150Thr NiR; (○) Met150Gly NiR; (●) wt NiR. Experimental conditions: $T = 298$ K, pH $= 7.0$, [NiR] $= 10\ \mu$M, and $I = 0.2$ M.

sites (Table 2, footnote b) and the values of K_{eq} obtained with eq 3, the values for the midpoint potential of the type-2 site could be calculated (average 234 ± 4 mV; see Table 2).

The second-order rate of reduction of wt NiR by Fe(II)-HEDTA and Fe(II)EDTA was not significantly influenced by the presence of acetamide (Table 2). The second-order rate constants for the reduction of Met150Thr, Met150Gly, wt NiR, and their known type-1 midpoint potentials (Table 2) were used to calculate $\Delta\lambda$ with eq 4. For Met150Gly and Met150Thr, with Fe(II)EDTA as well as Fe(II)HEDTA as the electron donor, $\Delta\lambda$ amounts to 310 ± 40 meV (30 ± 4 kJ mol^{-1}) (Table 2). When acetamide was added to Met150Gly NiR the rate of reduction decreased to slightly below that of the wt NiR (Table 2). Since for Met150Gly with acetamide bound the midpoint potential is < 225 mV versus NHE,[14] only an upper limit for $\Delta\lambda$ was obtained in the range of from 50 to 90 meV.

Internal Electron Transfer in NiR. Pulse radiolysis studies (Figure 3) were used as an independent method to determine the reorganization energies of the type-1 sites in wt NiR and Met150Gly, Met150His and Met150Thr NiR. The data were analyzed as described elsewhere.[6] In all NiR variants, the type-1 Cu(II) site was reduced in a bimolecular diffusion-controlled reaction by the 1-methylnicotinamide radicals produced in the pulse, after which redox equilibrium between the type-1 and the type-2 site was established (Figure 3). From this equilibrium the driving force, $-\Delta G°$, was calculated. The rates depended on the mutant (Table 3). From each rate, k_{298}, in combination with $-\Delta G°$ the total reorganization energy, λ_{Total} [$\lambda_{Total} = (\lambda_{T1} + \lambda_{T2})/2$, with λ_{T1} and λ_{T2} as the reorganization energies of the type-1 and type-2 sites] was calculated.[6] By using for λ_{T2} a value of 1.67 eV finally λ_{T1} was found.[6] The data are gathered in Table 3. The reorganization energies of the type-1 site were calculated to be significantly higher for the mutants (Table 3) than for the wt NiR. Within error the $\Delta\lambda$ values were equal to the 0.3 eV calculated from the stopped-flow experiments.

Crystal Structures of the Reduced NiR. Examination of all three monomers in the crystal structure of reduced Met150Gly-AcM shows that the Met62 side chain is displaced by acetamide and coordinates to the type-1 copper at a position that partly coincides with that of Met150 in wt NiR (Figure 4). The A chain of the structure was used for detailed comparison with oxidized Met150Gly-AcM.[14] Torsional changes in the Met62 side chain of reduced Met150Gly-AcM equal those in the oxidized structure. The χ_1, χ_2, and χ_3 angles change by 117°, 44°, and 20° respectively, compared to the Met62 side chain of reduced wt NiR. Additionally, the backbone φ angle shifts 17°. The combined torsional changes result in movement of the Met62 S_δ by roughly 4.4 Å to lie 2.28 Å from the type-1 copper atom. The acetamide molecule forms hydrogen bonds with two buried water molecules near the type-1 site (not shown).

Comparison of reduced Met150Gly-AcM with oxidized Met150Gly-AcM reveals small differences in coordination geometry (Table 4). Specifically, the Met62 S_δ−Cu bond is consistently shorter (~0.1 Å) and the Met62 S_δ shifts ~0.3 Å such that the Met62−Cu−His95 angle changes by 9° and the Met62−Cu−His145 angle changes by 5°. The conformation of Met62 differs slightly between the reduced and oxidized structures (rms deviation 0.16 Å). In contrast, the bound acetamide is well ordered (*B*-factor 36 $Å^2$) and is located at nearly the same position in either oxidation state (Figure 4).

The reduced wt NiR structure was solved to higher resolution (1.85 Å) than previously.[21] The observed differences at the type-1 site between reduced wt NiR and the oxidized structure are minimal (Figure 4, Table 4). Comparison of the ligand geometry over the three monomers in the asymmetric unit reveals a consistent lengthening of the His145−Cu bond in the reduced protein. This change is largely due to a shift in the

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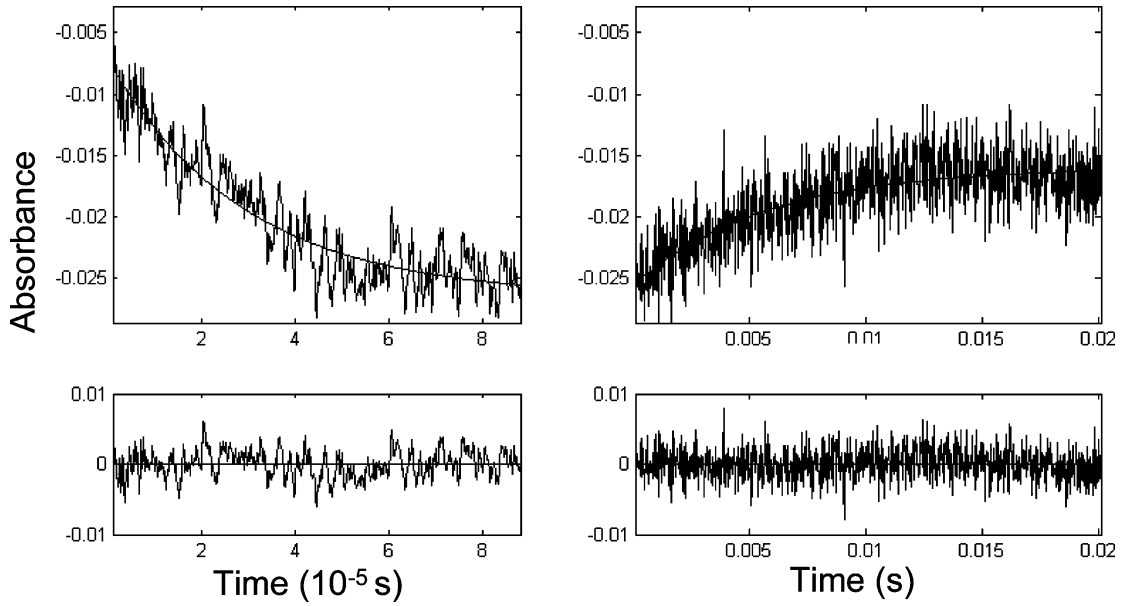

Figure 3. Time-resolved absorbance changes in pulse-radiolysis experiments. Reduction (left panel) and reoxidation (right panel) of the type-1 site in Met150Gly NiR as monitored optically at 460 nm (protein concentration 64 μM, pH 7.0, optical path length 3 cm, pulse width 0.5 μs). The *x*-axis represents time in seconds, while the absorbance is plotted on the *y*-axis. The fits are exponentials, and the residuals are given in the lower panels.

Table 3. Driving Force and Rate Constants for the Internal Electron Equilibration of NiR wt and Variants as Obtained by Pulse Radiolysis

variant	k_{298}[b] (s^{-1})	$-\Delta G^{\circ c}$ (meV)	k_2 (s^{-1})	k_{-2} (s^{-1})	λ_{Total}[d] (eV)	λ_{T1}[e] (eV)	$\Delta\lambda_{T1}$ (eV)
WT	2480 ± 160	-21	1696 ± 73	784 ± 233	1.12 ± 0.13	0.57 ± 0.26	0
Met150Gly	380 ± 30	78	18 ± 4	362 ± 34	1.39 ± 0.11	1.11 ± 0.22	0.54 ± 0.22
Met150His	2350 ± 170	-129	2333 ± 163	17 ± 7	1.29 ± 0.14	0.91 ± 0.29	0.34 ± 0.29
Met150Thr[a]	690 ± 40	106	11 ± 3	679 ± 43	1.25 ± 0.10	0.83 ± 0.20	0.26 ± 0.20

[a] The values for Met150Thr were determined at 275 K instead of at 298 K. [b] $k_{298} = k_2 + k_{-2}$ at 298 K. [c] 1 eV = 96.5 kJ mol^{-1}. [d] λ_{Total}, average reorganization energy of type-1 and type-2 site. [e] λ_{T1}, reorganization energy of the type-1 site.

copper position of roughly 0.1 Å in line with the lengthening of the His145−Cu bond.

Discussion

Reduction of NiR by Fe(II)EDTA/Fe(II)HEDTA. Stopped-flow data were analyzed on the basis of eqs 2 and 3 with a purpose written routine under Igor Pro (WaveMetrics, Inc.; available on request). Since Fe(II)HEDTA and Fe(II)EDTA are far larger than the physiological substrate (NO_2^-), they are not expected to enter the narrow tunnel[12,21,22] to the type-2 site cavity, as the SO_2^- radical can do in *Alcaligenes xylosoxidans* NiR.[23] The presence of a subsequent rapid equilibrium is confirmed by the rates measured by pulse radiolysis (Table 3); these rates are much faster than the rates of reduction of the type-1 site by Fe(II)EDTA/Fe(II)HEDTA.

The midpoint potential found for the type-2 site (234 ± 4 mV versus NHE) is within the range of midpoint potentials of type-2 sites of other nitrite reductases (172 mV to 310 mV[24,25]). In agreement with earlier findings[17] we observe that mutations in the type-1 site do not affect the type-2 site midpoint potential.[17] The data indicate that the rate of the second reduction step (the reduction of $T1^{OX}$;$T2^{RED}$) is identical to the rate of the first reduction step. This is similar to what is observed for *A. xylosoxidans* NiR.[23]

Determination of $\Delta\lambda$. For the estimation of $\Delta\lambda$ from stopped-flow experiments, eq 4 was used. The work terms and mechanistic details are assumed to be identical for the mutant and the wt proteins because the mutations are not located on the protein surface and do not introduce charges. As a check two different electron donors were used, $Fe(II)HEDTA^{1-}$ and $Fe(II)EDTA^{2-}$, which differ in coordination and net charge. They gave similar $\Delta\lambda$ values for ligand-free Met150Gly. Much smaller but again similar values were found for Met150Gly with acetamide (Table 2). Furthermore, the Met150Thr variant, with a different midpoint potential, showed the same $\Delta\lambda$ while the second-order rate for the reaction with Fe(II)HEDTA was different. Thus, it appears that the mutations indeed only affect midpoint potential and reorganization energy.

In a second series of experiments, the reorganization energies of the type-1 sites were measured by pulse radiolysis. Again an increase of λ was found for the NiR variants, within error equal to the values obtained by stopped-flow measurements (Table 3). Thus, both techniques report a substantial increase in λ due to the mutations.

Reorganization Energy and the Methionine Ligand. The reorganization energy is the energy needed to displace the nuclei from their equilibrium positions in the reduced state to the

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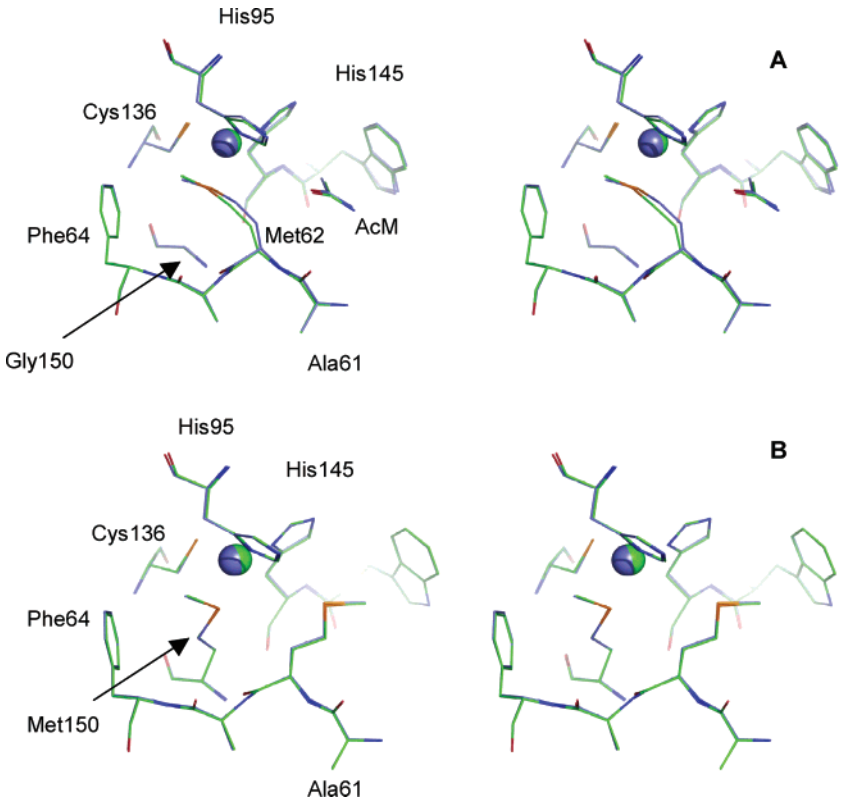

Figure 4. Stereoviews of Met150Gly-AcM and wt NiR. (A) Superposition of oxidized (green) and reduced (blue) Met150Gly-AcM. Residues near the type-1 site are labeled (AcM; acetamide). (B) Superposition of oxidized (PDB:1SJM, green) and reduced (blue) wt NiR. The copper atom is depicted as a sphere.

Table 4. Metal Ligand Geometry of Type-1 Sites in wt and Met150Gly NiR[a]

	oxidized wt NiR	reduced wt NiR	oxidized Met150Gly acetamide	reduced Met150Gly acetamide
		Distances (Å)		
axial−Cu	2.48	2.41	2.37	2.28
95−Cu	2.07	2.06	2.13	2.18
136−Cu	2.22	2.21	2.24	2.27
145−Cu	2.06	2.18	2.04	2.12
Cu−NSN[b]	0.64	0.66	0.63	0.70
		Angles (deg)		
136−Cu−95	129	130	126	119
136−Cu−Axial	106	109	100	100
Axial−Cu−95	89	90	95	104
Axial−Cu−145	133	131	129	124
θ[c]	64	64	71	78

[a] The numbers 95, 136, and 145 in the left column refer to the N_δ of His95, the S_γ of Cys136, and the N_δ of His145. Axial refers to the S_δ of Met150 in the wt NiR and the S_δ of Met62 in the Met150Gly structures. Sigma values (standard deviations determined from average values of three monomers in the asymmetric unit) amount to less than 5% for bond angles and less than 3% for bond distances. The data of reduced Met150Gly acetamide are those of the A chain which appeared to have least formation of a dinuclear site. [b] This is the distance between the Cu atom and the NSN plane determined by the ligand atoms of residues H95/Cys136/His145.[c] θ is the dihedral angle between the planes through Cu−136−Axial and the plane through Cu−95−145.

positions they occupy at equilibrium in the oxidized state without changing the redox state, and vice versa.[1] The reorganization energy can be divided into inner-sphere (displacing atoms belonging to the redox center) and outer-sphere (comprising surrounding atoms and solvent molecules) contributions. The outer-sphere reorganization energy is mainly due to the reorientation of the solvent and is effectively decreased by burying the redox center in the protein. Although an increase in the outer-sphere contribution to $\Delta\lambda$ in Met150Thr and Met150Gly cannot be excluded, it is unlikely to be significant as both mutants have an identical $\Delta\lambda$ (Table 2), while an identical increase in solvent accessibility is not expected with these mutations.

The inner-sphere reorganization energy (λ_i) can be calculated[1] from eq 5, in which ΔR_j is the change in bond length between Cu and ligand j upon oxidation, and k_j^{ox} and k_j^{red} are the corresponding force constants in the oxidized and reduced state of the Cu, respectively.

$$\lambda_i = \sum_j \frac{k_j^{ox} k_j^{red}}{k_j^{ox} + k_j^{red}} \Delta R_j^2 \qquad (5)$$

Equation 5 shows that a redox protein can lower λ_i by (a) decreasing the changes in bond lengths and (b) lowering the force constants.[26−29] From computational investigations[30−37] it was concluded that the presence of a methionine may lead to a

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reduction of λ_i by hundreds of meV. This is in line with the present experimental results which show that the binding of methionine lowers the reorganization energy of the type-1 site by 0.3 eV.

Lowering the reorganization energy by 0.3 eV has a considerable effect on the rate of electron transfer. Earlier it was observed[38,39] that site-directed mutagenesis of a naturally occurring axial ligand to a different naturally occurring axial ligand has less effect on the reorganization energy (0.1 eV). Apparently, native axial ligands decrease the reorganization energy to a similar extent.

Conclusions

A method employing small molecules was used to study the $\Delta\lambda$ that results from replacing a ligand methionine in the type-1 Cu site by a different amino acid. Crystal structures showed that the conformation of the type-1 site is hardly altered when NiR Met150Gly is reduced with an allosteric ligand in place. The same is observed for wt NiR. Furthermore, it makes no difference for the reorganization energy whether Met150 in wt NiR or Met62 in NiR Met150Gly binds to the copper. The binding of a methionine in a type-1 copper site lowers the reorganization energy by 0.3 eV and thereby promotes efficient biological electron transfer.

The technique used here to determine changes in reorganization energy is applicable to proteins with identical surface characteristics, such as appropriately engineered variants, and, for some applications, is a preferable alternative to NMR based techniques.[38,40]

Acknowledgment. This work was funded by an NSERC Discovery Grant to M.E.P.M. A research grant from the Minerva Foundation (Munich, Germany) to I.P. is gratefully acknowledged.

Supporting Information Available: A derivation of eqs 2 and 4 is presented, together with a brief discussion of conditions that lead to a simplified eq 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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