Allosteric control of internal electron transfer in cytochrome *cd*₁ nitrite reductase

Ole Farver^{*†}, Peter M. H. Kroneck[‡], Walter G. Zumft[§], and Israel Pecht[¶]

^{*}Department of Analytical Chemistry, Danish University of Pharmaceutical Sciences, DK-2100 Copenhagen, Denmark; [‡]Fachbereich Biologie, Universität Konstanz, D-78457 Konstanz, Germany; [§]Lehrstuhl für Mikrobiologie, Universität Fridericiana, D-76128 Karlsruhe, Germany; and [¶]Department of Immunology, Weizmann Institute of Science, 76100 Rehovot, Israel

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Cytochrome *cd*₁ nitrite reductase is a bifunctional multiheme enzyme catalyzing the one-electron reduction of nitrite to nitric oxide and the four-electron reduction of dioxygen to water. Kinetics and thermodynamics of the internal electron transfer process in the *Pseudomonas stutzeri* enzyme have been studied and found to be dominated by pronounced interactions between the *c* and the *d*₁ hemes. The interactions are expressed both in dramatic changes in the internal electron-transfer rates between these sites and in marked cooperativity in their electron affinity. The results constitute a prime example of intraprotein control of the electron-transfer rates by allosteric interactions.

Site-site interactions are central to regulatory mechanisms used by proteins. Although numerous examples of this activity exist in enzymes, receptors, and transport proteins, allosteric regulation of electron transfer (ET) in redox enzymes has rarely been addressed, and no kinetic analysis of such processes has so far been attained. Here we report on the allosteric control of electron distribution and transfer rates between the heme sites of cytochrome *cd*₁ nitrite reductase (*cd*₁ NiR; EC 1.9.3.2) from *Pseudomonas stutzeri* (*Ps*) (1). This enzyme is a homodimer of ≈60-kDa subunits, each containing a covalently bound heme-*c* and a noncovalently bound *d*₁-type heme. It catalyzes the one-electron reduction of nitrite to nitric oxide as well as the four-electron reduction of dioxygen to water (1, 2). *cd*₁ NiR thus catalyzes the first committed step in dissimilatory nitrite reduction, leading to dinitrogen, fulfilling a key role in geochemical nitrogen transformations and in balancing the assimilatory branch of the global nitrogen cycle (1). Earlier potentiometric and spectrophotometric titrations have suggested that cooperativity prevails in the interactions among heme sites in *cd*₁ NiRs isolated from *Pseudomonas aeruginosa* (*Pa*) (3) and *Paracoccus pantotrophus* (*Pp*) (4), yet the studies above failed to address their kinetic basis. Although all three enzymes show a marked homology in their amino acid sequences and, for the *Pp* and *Pa* proteins, also similarity in three-dimensional structures, some noteworthy and intriguing structural differences have been observed that may imply significant differences in functional behavior (1, 5–8): Heme-*c* Fe(III) in *Pp-cd*₁ NiR has His/His axial ligands, whereas at the heme-*d*₁ Fe(III) the axial ligands are Tyr/His (5). On reduction, the heme-*c* Fe(II) ligands switch to His/Met concomitant with dissociation of the tyrosine ligand leaving the heme-*d*₁ Fe(II) penta-coordinated (7). In contrast, in *Pa-cd*₁ NiR heme-*c* is His/Met coordinated in both oxidation states, whereas the axial heme-*d*₁ ligands are hydroxide and His in the oxidized state and assumed to become penta-coordinated (vacant/His, respectively) on reduction (8). A remarkable feature of *Pa-cd*₁ NiR enzyme is the “arm exchange” or “domain swapping” of its N-terminal tail that places Tyr-10 of one monomer close to the heme-*d*₁ site of the other one. Tyr-10 is hydrogen-bonded to the heme-*d*₁ hydroxide ligand, thereby preventing access of the substrate to the catalytic site (6). In contrast, no “domain swapping” occurs in *Pp-cd*₁ NiR, and Tyr-25 of the *c*-domain coordinates directly to the heme-*d*₁ iron of the same monomer

(5). The N-terminal tail of *Ps-cd*₁ NiR differs markedly from those of the other two enzymes suggesting a different mode of interaction. Therefore, we have now studied both the thermodynamics and kinetics of internal ET in the *Ps* enzyme by pulse radiolytically produced *N*-methylnicotinamide radicals.

Materials and Methods

Cytochrome *cd*₁ from *P. stutzeri* strain ZoBell (ATCC 14405) was purified, and its biochemical and spectroscopic parameters were characterized as described (9). Pulse radiolysis experiments were performed on the Varian V-7715 linear accelerator of the Hebrew University in Jerusalem (10). Electrons accelerated to 5 MeV were used with pulse lengths in the range from 0.1 to 1.5 μs and introduced into argon-saturated solutions containing 5 mM *N*-methylnicotinamide, 5 mM phosphate, 0.1 M *tert*-butanol, pH 7.0. All optical measurements were performed anaerobically under purified argon at a pressure slightly in excess of 1 atm (1 atm = 101.3 kPa) in a 1- or 3-cm Spectrosil cuvette. The reduction states of both heme types were monitored independently by measuring time-resolved absorption changes at 554 nm (heme-*c*) and 640 nm (heme-*d*₁). Two distinct time bases were used in our time-resolved measurements, and absorbance changes were fitted to a sum of exponentials by using a nonlinear least-squares program written in MATLAB. Three exponentials were typically used in analyzing heme-*c* data: one for the fast bimolecular step and two for the slower reoxidation of the individual species (see below). For analysis of the intramolecular heme-*d*₁ reduction two exponentials were sufficient. The fit was not significantly improved by adding extra exponentials.

A sequence of single pulses was applied to each protein solution, eventually leading to full reduction of the enzyme. Each experiment was analyzed separately. A simulation program that includes all 10 different possible redox states of the enzyme was written in MATLAB. The simulation procedure was applied in 40 steps of 0.1 electron equivalents, and each of these includes initial electron uptake by heme-*c*(III) and internal electron redistribution.

The internal electron equilibration between heme-*c* and -*d*₁ was evaluated from the amplitudes of the 554- or 640-nm time-resolved signals. The initial reduction-phase amplitudes provided the total electron uptake through heme-*c*. Amplitudes of the ensuing absorbance changes allowed for calculation of the relative distribution between the different species present during a series of pulses, and determination of the constants of the four individual equilibria. Because *K*₁ has already been determined (11) this parameter is kept fixed in the fitting procedure, whereas the other three intrinsic constants are allowed to vary. This way, the *K*₂ to *K*₄ values that give the best fit to the observed amplitude changes are determined. The rate constants for the individual steps, *k*_{fi} +

Abbreviations: ET, electron transfer; *cd*₁ NiR, cytochrome *cd*₁ nitrite reductase; *Ps*, *Pseudomonas stutzeri*; *Pa*, *Pseudomonas aeruginosa*; *Pp*, *Paracoccus pantotrophus*.

[†]To whom correspondence should be addressed. E-mail: of@dfh.dk.

Fig. 1. (A) Observed rate constants of intramolecular heme-*c*-heme *d*₁ ET. ■ (554 nm) indicates heme-*c* reoxidation and ● (640 nm) denotes heme-*d*₁ reduction. Temperature, 30.5°C; pH 7.0. (Inset) Calculated distribution of the different species produced as a function of the enzyme's reduction state. Numbers 1 and 10 represent fully oxidized and fully reduced enzyme molecules, respectively. The other numbers refer to partially reduced molecules as defined in Scheme 1. Species 2 and 3 are in equal concentrations ($K_{298} = 1.0$) and therefore are superimposed on each other in the graph. Number 6 represents molecules in which heme-*c* and heme-*d*₁ in one subunit are both reduced, whereas the hemes of the other subunit are in the oxidized state. Thus, this species cannot be involved in internal ET. (B) The points represent ratios between observed amplitudes of heme-*c* reoxidation and heme-*c* reduction, respectively, after each pulse. In each experiment, the amplitude of the fast heme-*c*(II) reduction is a measure of the number of reduction equivalents added to the enzyme in this particular pulse (A_{tot}). Part of heme-*c*(II) is then reoxidized by the internal ET to heme-*d*₁(III) (A_{reox}). The remaining part of the reduced heme-*c*(II) is A_{red} . Thus, for each pulse $A_{\text{tot}} = A_{\text{reox}} + A_{\text{red}}$, and $R(\text{reox/red}) = A_{\text{reox}}/A_{\text{red}}$. The points have been plotted against the total number of reduction equivalents added to the enzyme at each step of the pulse radiolytic reduction titration. The extended line was calculated from the model using the equilibrium constants given in Scheme 1. All experimental conditions were the same as in A.

k_{b1} , etc. were then calculated from the changes in observed rate constants following the number of electron equivalents taken up by the enzyme (cf. Fig. 1A).

Results and Discussion

The reduction states of both heme types were monitored independently by measuring time-resolved absorption changes at 554 nm (heme-*c*) and 640 nm (heme-*d*₁). The initial process monitored after the pulse is a bimolecular reaction where heme-*c* is reduced by the radicals (11). This process is followed by an internal unimolecular electron transfer to the *d*₁-heme, which was always slower and well separated from the initial bimolecular step. Introducing sequential pulses into solutions of the enzyme under exclusion of dioxygen resulted in accumulation of reduction equivalents in the heme sites, eventually adding up to four, leading to a fully reduced enzyme. We had earlier examined this internal ET process under conditions where up to one reduction equivalent only was introduced into the enzyme and determined equilibrium and activation parameters of this step (11). Proceeding now with the reduction and adding more than two electron equivalents caused the internal *c* to *d*₁ ET rates to decrease by more than two orders of magnitude, which is illustrated in Fig. 1A, showing the intramolecular ET rate dependence on the degree of enzyme reduction. Similarly, the internal electron distribution between the *c* and *d*₁ heme sites in each monomer depended on the number of reduction equivalents taken up by the enzyme (Fig. 1B). The same pattern was observed over the whole temperature range examined (3–40°C).

The hemes' mutual interaction dependence on the degree of the enzyme-reduction state has been analyzed by using a model that involves electron uptake by the *c* hemes followed by equilibration between hemes-*c* and -*d*₁ within the same subunit. Intersubunit ET equilibration has been ignored because the heme-heme separation distances in the dimer are too large to allow its occurrence during the examined time domain and so were also intermolecular ET between enzyme dimers (5, 6). Results of these calculations are presented by the extended line in Fig. 1B and in Scheme 1. The model described in the scheme includes only the four equilibria in which intrasubunit ET can take place.

Standard enthalpy and entropy changes for the intraprotein ET equilibrium constants were further determined: ΔH^0 (kJ·mol⁻¹) = -24.9 ± 2.5 , $+124 \pm 20$, -113 ± 25 , and -43 ± 13 , and ΔS^0 (J·K⁻¹·mol⁻¹) = -83 ± 8 , $+436 \pm 65$, -400 ± 68 , and -122 ± 45 . The exceptionally large changes in both enthalpy and entropy probably reflect distinct mechanisms operating in the different steps, e.g., involving a conformational transition.

The calculated detailed internal electron distribution equilibria exhibit large changes on proceeding from one reduction state to the other (Scheme 1 and Fig. 1A Inset). Although, with a single electron per enzyme dimer, equidistribution prevails, adding a second equivalent causes preference for the asymmetric distribution (species 5).

As K_2 and K_3 clearly show, equilibrium distribution among species 4, 7, and 5 is markedly shifted toward the latter, i.e., molecules where one heme site (*c* or *d*₁) is reduced prevail. Introducing the third electron shifts the equilibrium in the opposite direction causing predominance of species where both hemes *d*₁ (species 9) are reduced, as K_4 shows. The parallel marked changes in kinetic parameters are also interesting. A conspicuous drop is seen in the rate of internal heme *c*[Fe(II)]-heme *d*₁[Fe(III)] ET after adding the second electron equivalent to the enzyme. The observed intramolecular rate constant is an average value weighted by the amplitudes of the different species present (cf. Fig. 1A). This follows from analysis of the intramolecular electron distribution where the observed change corresponds to the first two forward rates being much faster than the latter two. The above-mentioned decline in the internal ET rate provides clear kinetic evidence for negative cooperativity between the two heme-*d*₁ sites.

Scheme 1. Intramolecular heme-*c* to heme-*d*₁ ET equilibration. The scheme includes only those species among which intramolecular electron equilibration can take place. White symbols represent oxidized hemes and gray symbols represent reduced ones. The equilibrium distribution parameters were calculated from the rates and amplitudes at different degrees of reduction (see Fig. 1). The error range for the parameters of step 1 is $\pm 10\%$. For the remaining steps, the error range is $\pm 20\%$.

Moreover, the agreement between the calculated electron distributions and rate constants obtained by using a model based on this negative cooperativity and the experimental data strongly support the appropriateness of the model used.

The specific rates observed for the individual internal ET steps (Scheme 1) deserve attention. Assuming that the distance between the iron centers of the two hemes in one subunit is the same as found in *Pa* and *Pp* NiR (20.6 Å) (5–8) we can calculate the expected species 2 to species 3 intramolecular ET rate in the activationless case, $k_{\text{MAX}} = 10^4 \text{ s}^{-1}$, by using the procedure outlined by Gray and Winkler (12). The experimentally observed rate constant is 11 s^{-1} , with zero driving force ($K = 1.0$ at 298 K) (11). By using this value we may calculate a reorganization energy for heme-*c* to heme-*d*₁ ET, $\lambda = 0.7 \text{ eV}$ (67 kJ mol⁻¹), which is in the range expected for heme reorganization (0.8 eV) (12). From Scheme 1 it is obvious that forming species 5 from the half-reduced species 4 and 7, respectively (where either both hemes-*c* or both hemes-*d*₁ are fully oxidized) proceeds with essentially the same driving force ($\approx 0.08 \text{ eV}$). Thus, the 50-fold difference in rate constant precludes changes in the driving force being of major

influence. So are changes in reorganization energy because this would require an increase in λ of no less than 0.3 eV, which is quite unrealistic. Hence, this decrease in rate must be due to other changes taking place in the enzyme at that stage, probably in structure. Redox-induced conformational changes have been reported for both *Pa*- and *Pp*-*cd*₁ NiRs (5–8, 13). Related conformational changes are likely to occur in *Ps*-*cd*₁ NiR and may provide a rationale for the steep decline in rates. Specifically, a structural change reducing the electronic coupling between donor and acceptor would cause a marked decrease in ET rate constant; e.g., breaking one hydrogen bond forcing a through-space jump across the same 0.28-nm distance could account for an ≈ 50 -fold drop in rate constant. Such rate modulation caused by intrinsic properties of the protein provides an interesting illustration for “gating” of ET reactions.

A key question raised by the current results is why has evolution selected such an elaborate control mechanism for an enzyme catalyzing a relatively simple one-electron-transfer process? This question becomes even more challenging because some bacteria carry a different type of nitrite reductase containing type 1 and 2 copper sites as active centers that do not show the above-mentioned allosteric control (14).

One possible rationale for this question is pertinent to the nitrite reduction mechanism of this enzyme as the observed electron distribution pattern minimizes the probability of product inhibition by NO binding to a low spin heme-*d*₁ Fe(II), which has a high affinity for this molecule. This is obvious for species 5 in Scheme 1 and for all other forms of the enzyme reduced to a higher degree; a 50-fold or more slower internal ET rate will enable NO dissociation before re-reduction of heme-*d*₁.

Another explanation may be in the fact that the *cd*₁ NiRs reflect the bacterial adjustability to changing environments as they have evolved under intermittently anaerobic and facultatively aerobic conditions. The physiological role of the dioxygen reduction capacity of *cd*₁ NiR is not fully understood. Several such enzymes are usually present in a bacterium and the systematic knockout mutagenesis of oxygen reductase activities, including cytochrome *cd*₁ to reveal its contribution to cellular oxygen metabolism, has not yet been performed. Still, one might propose that the above-described site–site interactions have evolved for this process.

In conclusion, the present results provide a prime example for a built-in control mechanism of intraprotein ET reactivity. It therefore constitutes an attractive model for pursuing the challenge of how internal control of charge migration and distribution takes place in one of nature’s key players in biological energy conversion, cytochrome *c* oxidase (15).

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