FEBS Letters xxx (2011) xxx–xxx

journal homepage: www.FEBSLetters.org

Review

Gating mechanisms for biological electron transfer: Integrating structure with biophysics reveals the nature of redox control in cytochrome P450 reductase and copper-dependent nitrite reductase

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ARTICLE INFO

Article history:
Received 1 June 2011
Revised 1 July 2011
Accepted 4 July 2011
Available online xxxx

Edited by Miguel Teixeira and Ricardo O. Louro

Keywords:
Electron transfer
Gating
Proton coupled electron transfer
Conformationally controlled electron transfer

ABSTRACT

Biological electron transfer is a fundamentally important reaction. Despite the apparent simplicity of these reactions (in that no bonds are made or broken), their experimental interrogation is often complicated because of adiabatic control exerted through associated chemical and conformational change. We have studied the nature of this control in several enzyme systems, cytochrome P450 reductase, methionine synthase reductase and copper-dependent nitrite reductase. Specifically, we review the evidence for conformational control in cytochrome P450 reductase and methionine synthase reductase and chemical control i.e. proton coupled electron transfer in nitrite reductase. This evidence has accrued through the use and integration of structural, spectroscopic and advanced kinetic methods. This integrated approach is shown to be powerful in dissecting control mechanisms for biological electron transfer and will likely find widespread application in the study of related biological redox systems.

1. Introduction

Electron transfers are key biochemical events catalysed by biological systems. They are ubiquitous and important reactions, but can be difficult to study. Unlike most heavy atom transfers, electron transfers can occur over large distances (~15 Å). Electron transfer is understood in the context of the Marcus formalism which explicitly recognises the importance of reorganisation energy [1]. Electron transfer presents several challenging problems that must be overcome by enzymes in order to efficiently catalyse a given reaction. Given the potentially large transfer distance of an electron, coupled with the fact that chemical bonds may not be broken or formed, the reaction coordinate may be difficult to define optimally. Further, the ideal reaction coordinate may correlate poorly with the optimal binding geometry of substrate molecules. It is apparent that a general strategy may be to couple conformational change, or other chemical events, to biological electron transfer to achieve meaningful catalysis. Often enzyme-catalysed electron transfer may be far slower than would be predicted by the Marcus formalism. Such adiabatic reactions are termed 'gated electron transfers', defining the situation where other mechanistic steps influence the rate of electron transfer. Conformational change may be used to alter driving force, reorganisation energy or electronic coupling to control electron transfer dynamically. Alternatively, electron transfer may be coupled to chemical steps giving another mechanism of control. It is possible to identify such gated electron transfer reactions, for example by monitoring the temperature dependence of the reaction rate, and assessing any deviation from Marcus theory [2]. However, it is often difficult to identify whether gating contributions arise from chemical or conformational events or even the availability of protons. In the present article we discuss enzyme model systems, Cu-dependent nitrate reductase (NiR), cytochrome P450 reductase (CPR) and the related human methionine synthase reductase (MSR), which exemplify chemical and conformational gating of their respective electron transfer reactions. These model systems illustrate the benefit of integrating multiple experimental

Abbreviations: *Ax*NiR, *Alcaligenes xylosoxidans* nitrate reductase; CPR, cytochrome P450 reductase; CYP, cytochrome P450; PELDOR, pulsed electron–electron double resonance; ENDOR, electron nuclear double resonance; PCET, proton coupled electron transfer; SKIE, solvent kinetic isotope effect; T1Cu, type 1 copper; T2Cu, type 2 copper

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doi:10.1016/j.febslet.2011.07.003

approaches to dissect in detail the role of both chemical and conformational gating in biological electron transfer.

2. Conformationally gated electron transfer in CPR

CPR is a membrane-bound NADPH-dependent oxidoreductase that contains FAD and FMN cofactors housed in discrete redox domains separated by a flexible loop region [3]. CPR catalyses electron transfer from NADPH to cytochrome P450 (CYP) enzymes in the endoplasmic reticulum. The chemical mechanism of electron transfer is relatively well understood [4, 5]. NADPH binds to the FAD domain where it transfers a hydride to the N5 of FAD followed by electron transfer from FAD to FMN to yield a thermodynamic distribution of 2-electron reduced species ($FADH^{\cdot}$ $FMNH^{\cdot}$, $FADH_2$ FMN and FAD $FMNH_2$). In the absence of an electron acceptor (such as a cognate cytochrome P450 enzyme, CYP) a second equivalent of NADPH binds to the FAD domain and transfers a hydride to FAD driving the equilibrium distribution of enzyme states towards the fully (4-electron) reduced species ($FADH_2$ $FMNH_2$).

X-ray crystal structures suggest that the inter-flavin distance is short (~4 Å) implying that electron transfer should be fast ($\sim 10^{10}\ s^{-1}$) based on distance arguments alone [6]. However, despite this proximity, inter-flavin electron transfer is slow ($\sim 50\ s^{-1}$) as measured by temperature jump [7, 8] and flash photolysis [9] time-resolved spectroscopies. The observed rates of inter-flavin electron transfer have significant ionic strength and viscosity dependencies [4], which is not expected for non-adiabatic 'true' electron transfer, implying adiabatic control of the reaction [10]. A large body of evidence implicates conformational control of electron transfer in CPR. This has been the focus of numerous studies aimed at understanding the molecular details of conformational control, which have integrated structural, spectroscopic and kinetic studies. In this review, we discuss a number of key experimental methods that have been used to support the notion of conformational gating in CPR. Integration of these approaches has significantly advanced our knowledge of dynamical control in CPR and could similarly find application in other systems where dynamics is thought to contribute to mechanism.

2.1. X-ray crystallography

Crystallographic evidence for conformational variation has accumulated over the years for rat CPR, which is structurally similar to human CPR (94% sequence identity) [3, 11–13]. These structures show several different structural states of CPR, ranging from 'closed' structures where the dimethylbenzene moieties of the FAD and FMN are juxtaposed, to 'open' structures with dramatically increased inter-flavin distances. Reorientation of the two flavin-binding domains appears to arise via a connecting loop, which acts as a "hinge" (Fig. 1). A CPR variant where four residues were deleted from this hinge region (ΔTGEE) retained essentially wild-type observed rates of electron transfer between CPR and CYP when fully reduced [12]. Several X-ray crystal structures of the ΔTGEE variant show a significantly extended (or open) structure compared to wild-type enzyme, whilst the structural integrity of the individual FMN/FAD-domains remains essentially unchanged. These data are in agreement with a model in which CPR can adopt more open conformations that are competent for interaction with CYP redox partner proteins. This model is consistent with other work which suggests residues required for interaction with CYPs are occluded in closed conformations of CPR [14]. While the inter-protein electron transfer rates of the ΔTGEE construct were unchanged as compared to the wild-type, ΔTGEE displayed impaired observed rates of electron transfer between the two flavin moieties [12]. This observation implies that the increased inter-flavin distance observed in the X-ray crystal structures of the ΔTGEE variant compromises the observed rate of electron transfer. Taken together, the kinetic data underpin the mechanistic relevance of the various conformations observed in the crystal structures. Similar findings have arisen from another study where deletion of the hinge region gives rise to a dramatic decrease in inter-flavin electron transfer [15]. Small angle X-ray scattering (SAXS) data suggests that the impaired electron transfer in the hinge deletion CPR variant may arise from impaired diffusional motion of the FAD and FMN domains which in turn prevents a favourable domain configuration for inter-flavin electron transfer [15].

A recent study supports the hypothesis of enzymatically relevant conformational dynamics in CPR. Xia et al. engineered a non-native disulphide linkage between the FMN and FAD domains of wild-type rat CPR [16]. The X-ray crystal structure shows that such a linkage gives rise to an artificially closed structure of CPR with a relatively short inter-flavin distance. Unlike with the artificially open ΔTGEE variant of CPR, the observed rates of electron transfer from CPR to CYP and the observed rates of inter-flavin electron transfer were found to be significantly impaired in this artificially closed form. Crucially, the reduction of CYP by CPR activity was rescued upon reduction of the disulphide linkage. Taken together, these data were rationalised by invoking the need to adopt more open conformations of CPR to interact optimally with partner CYP proteins. These data therefore offer support for a model in which open conformations are required to pass electrons to CYPs, but also indicate that the open structure compromises inter-flavin electron transfer in CPR. A dynamic model for CPR function would therefore

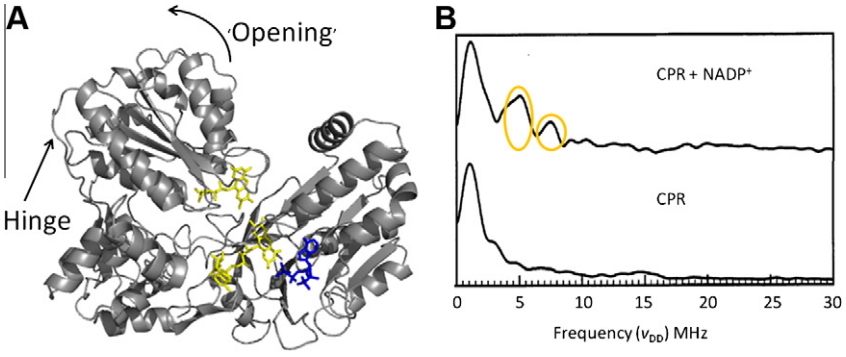

Fig. 1. (A) CPR adopts multiple conformations as observed from a series of X-ray crystal structures. The structure shown is the closed conformation with the flavin moieties shown in yellow and 2′, 5′-adenosine di-phosphate shown in blue. Opening is proposed to be mediated by a hinge region, which allows the FMN domain to move away from the FAD domain. (B) Adapted from ref [18]. Fourier-transformed PELDOR spectra of di-semiquinoid CPR with (upper) and without (lower) $NADP^+$ bound. The major peak at ~1.5 MHz in both spectra corresponds to the same inter-flavin distance. The highlighted new peaks in the $NADP^+$-bound spectra correspond to new conformations with shorter inter-flavin distances, suggesting that coenzyme binding allows formation of more closed states of CPR.

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need to control structural transitions (open to closed and vice versa) at different points in the catalytic cycle.

2.2. Magnetic resonance studies

NMR spectroscopy and SAXS data have provided further evidence for structural variation in CPR, particularly for the relevance of the closed state [17]. The ^{15}N–1H chemical shift differences were used to monitor variation in the FMN/FAD domain interaction. NMR data have suggested that CPR can exist in equilibrium between at least two conformations, one of which corresponds to the closed X-ray crystal structure. The authors also performed SAXS studies of CPR in different oxidation states, monitoring the scattering profile of both oxidised and 4-electron reduced CPR. The scattering curves were not super-imposable for the different oxidation states, suggesting a relationship between the oxidation state and conformational state of CPR. Shape reconstruction of the scattering profiles for oxidised and reduced CPR states gave low-resolution molecular envelopes for comparison to the available X-ray crystal structures. These data suggested again that CPR can adopt at least two experimentally observable conformational states. The SAXS data suggested a more closed form was favored on coenzyme binding to both the oxidised and reduced forms of CPR. That is, the binding of coenzyme induces a shift in the equilibrium of conformational states of CPR, or alternatively that coenzyme preferentially binds to the closed state of CPR.

Using pulsed electron–electron double resonance (PELDOR) techniques the inter-flavin distance in the 2-electron reduced (di-semiquinone) form of CPR was monitored [18]. This method gives a way of directly monitoring variation in the conformational state of CPR. The dipole–dipole coupling between the two electronic magnetic moments of the flavin semiquinone centres is related to the distance between them by Eq. (1).

$$\nu_{DD}(\theta, r) = \frac{g_1 g_2 \mu_0 \mu_B^2}{4\pi h} \frac{1}{r^3}(3\ \cos^2\ \theta - 1) \quad (1)$$

where g_1 and g_2 are the g values of the two spins, r is the distance between them, and θ is the angle between the inter-spin vector and the applied magnetic field. Using this method, the predominant inter-flavin distance for the di-semiquinoid CPR was found to be ~36 Å. Fourier transforms of the extracted time-traces suggest that binding of the NADPH coenzyme mimic NADP$^+$ causes the inter-flavin distance to decrease, but also gives rise to a broad population of distances ranging from the apo-enzyme value (~36 Å) to 19 Å (Fig. 1). These data correlate well with the NMR data discussed above, where coenzyme binding was found to give rise to more closed states of CPR. However, the PELDOR data indicate that coenzyme binding itself may result in a broad population of CPR conformations, and that the equilibrium distribution is shifted to more closed states. Interestingly, high ionic strength conditions lead to a similar shortening of inter-flavin distance over a relatively broad population of conformations. Taken together, the PELDOR studies suggest that CPR can adopt many conformations with potential implications for electron transfer. Specifically, the PELDOR results represent the conformational distributions exhibited by the protein just before motion ceased due to lack of thermal energy (samples are frozen at 80 K), i.e. those conformations that exist at the bottom of thermodynamic 'wells' or minima.

The related di-flavin enzyme methionine synthase reductase (MSR), is also amenable to PELDOR spectroscopy, since the di-semiquinone form of MSR can be stably formed [19]. MSR catalyses a similar reaction to CPR, where electrons are transferred from NADPH, ultimately to the acceptor enzyme methionine synthase (MS) [20]. A full-length crystal structure for MSR is lacking, but structural data are available for the isolated FAD/NADPH-binding domain [20]. As with CPR, PELDOR data suggest that NADP$^+$ binding induces a broad distribution of conformations with decreased inter-flavin distances [19]. In addition, electron nuclear double resonance (ENDOR) spectroscopy was used to monitor the hyperfine coupling between atomic nuclei and the unpaired electrons of the flavin semiquinones [19]. These data allow conformational variations in the flavin moieties themselves to be assessed. The ENDOR data dominantly represent the environment of the 8-methyl group of the flavin semiquinone dimethylbenzene ring. Variation in the hyperfine couplings was attributable to the 8-methyl group dynamics alone and not multiple geometries of the flavin moieties themselves. These data provide strong evidence that the broader distribution of conformational states observed in the PELDOR data is attributable to domain motion (resulting in more closed conformations) and not internal motion of the flavin cofactors. Further, these data suggest that conformational sampling of multiple reactive geometries may be a general feature of the function of di-flavin reductases. Data from PELDOR and ENDOR experiments are therefore informative, particularly in situations (as with MSR) where there is no available crystal structure for the full-length enzyme.

2.3. Transient state kinetics

Structural studies can inform on absolute changes in conformational state but it is more difficult to link these changes with defined mechanistic steps. In CPR, the redox-state dependent absorbance changes of the flavin cofactors allow use of anaerobic stopped-flow techniques to access steps broadly associated with the initial 2-electron reduction and then subsequent 4-electron reduction of the flavin moieties [4]. High pressure can be used to perturb the equilibrium of conformational states as demonstrated previously [21]. Given the apparently broad distribution of conformational states associated with the coenzyme-bound form of CPR as evidenced from PELDOR data [18], high pressure stopped-flow studies of the kinetics of inter-flavin electron transfer have been performed to probe the functional relevance of this conformational distribution [18]. Such an approach allows structural perturbation to be correlated with catalytic activity. The expectation was that increasing pressure would favour more compact conformations, leading to enhanced inter-flavin electron transfer, essentially giving rise to enhanced observed rates of 2- and 4-electron reduction. The observed rates for both 2- and 4-electron reduction were found to increase with increasing pressure as expected, though exhibiting different pressure-dependencies. The data suggest that CPR occupies a higher population of closed, electron transfer competent states at high pressure. In combination with the PELDOR data, the high-pressure study suggests that CPR can adopt multiple conformational states (particularly in complex with the coenzyme) and that these new states are functionally relevant. Further, these data support a hypothesis where inter-flavin electron transfer is at least partly controlled by conformational sampling, induced by the binding of coenzyme.

In summary, the studies highlighted here point to dynamic interchange between several conformational states that contribute to the overall function of CPR. Conformational sampling appears to play a key role in controlling the rate of inter-flavin electron transfer allowing access to more closed conformations with shorter inter-flavin distances. Such conformational sampling mechanisms may be induced by coenzyme binding itself. Further, the current model for CPR function implies that more open conformations can be populated and that these conformations are needed to interact with partner CYP enzymes. Work with CPR therefore demonstrates the power of integrating structural studies with advanced spectroscopy techniques (e.g. PELDOR) and analysis of reaction kinetics (e.g. as a function of temperature and pressure). Similar approaches will no doubt inform on conformationally gated electron transfer reactions in other model protein systems.

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3. Chemical gating of inter-Cu electron transfer in NiR

In many metalloenzymes such as cytochrome *c* oxidase, nitrogenase, nitrite reductases and hydrogenases, catalysis involves the controlled delivery of electrons and protons to the active site where substrate is utilised. Electron transfer is often coupled to proton transfer, and is frequently accompanied or induced by conformational change or by the binding of small molecule substrates. Metal proteins, however, often show only small structural changes around the metal atom upon reduction or removal of the metal [22], suggesting that mechanisms other than large-scale protein motions are at the basis of electron transfer control in metalloenzymes. While our understanding of factors involved in electron transfer is advanced, our knowledge of proton transfer and other coupled processes is less so.

The coupled transfer of electrons and protons is a common phenomenon in many important biological processes, such as photosynthesis, respiration and denitrification [23]. Thermodynamic coupling between electrons and protons allows pH changes to be used to induce electron transfer through films or over long distances in molecules and through proton channels in membranes [24]. Unlike hydrogen atom and hydride transfer, proton-coupled electron transfer (PCET) reactions typically involve different donors and acceptors for the electron and proton [25]. It is not the purpose of this review to provide details about the theoretical background of PCET, as excellent recent reviews are available [23, 24, 26, 27]. PCET processes have been identified and thoroughly studied in different redox enzyme types including cytochrome *c* oxidase [28], photosystem II (part of the oxygen evolving complex that catalyses the light-driven oxidation of water to oxygen) [29], and nitrite reductase, a key enzyme in microbial denitrification [30]. Here, we consider experimental evidence and approaches that have been used recently to demonstrate PCET in *Alcaligenes xylosoxidans* nitrite reductase (*Ax*NiR).

3.1. Probing inter-copper electron transfer in AxNiR by laser-flash photolysis

The use of laser-flash photolysis to rapidly inject electrons into redox enzymes is a powerful tool to study fast electron transfer reactions in multi-site redox enzymes that are not accessible using conventional rapid-mixing stopped-flow methods. Photoexcitation also circumvents the limitations imposed by mixing enzyme and substrate solutions to initiate the reaction, allowing the study of solvent and protein dynamics at high solution viscosities [31]. Various photoexcitable electron donors have been developed and successfully applied in different redox enzyme systems. Thioredopyrene-3-6-8-trisulfonate (TUPS) was used to study inter-flavin electron transfer in CPR and nitric oxide synthase (NOS) [32]. TUPS can be used both in solution and covalently attached to the protein via a surface cysteine. The same approach was used for ruthenium polypyridyl complexes with cytochrome *c* oxidase. The focus has been on electron transfer from the heme *c* in cytochrome *c* to Cu_A, the internal electron transfer from Cu_A to heme *a*, and subsequently to the heme a_3/Cu_B binuclear centre, as well as the coupled proton pumping. [33]. Laser-flash photolysis using NAD(P)H as electron donor has been successfully used to study heme reduction and ligand binding in various cytochrome P450 enzymes [34, 35], and the inter-copper electron transfer in *Ax*NiR [30].

NiRs catalyse the one-electron reduction of nitrite into gaseous nitric oxide (NO), the first committed step in microbial denitrification [36]. Cu-dependent NiRs are homotrimeric and receive electrons at their T1Cu site from a partner redox protein. The electrons are transferred, via a covalent His–Cys bridge, to the T2Cu site, where nitrite is reduced to NO. The electron transfer pathway between T1 and T2Cu is 12.6 Å long and consists of 11 covalent bonds comprising the T1Cu ligand Cys130 and the T2Cu ligand His129 (Fig. 2) [37]. Inter-Cu electron transfer in CuNiR has been studied using pulse radiolysis techniques, and electron transfer has been measured both in the absence and presence of substrate [38–40]. An extensive analysis using pulse radiolysis is, however, hampered by very small absorbance changes. We have investigated the nature of control of inter-Cu in *Ax*NiR using a different laser-flash photolysis procedure relying on photoexcitation of NADH [30].

Inter-Cu electron transfer in *Ax*NiR can be conveniently studied using laser-photoexcitation methods, because the T1Cu is located just below the protein surface and is much more readily reduced than the buried T2Cu centre. Because the T2Cu is effectively optically 'silent' in either reduction state, only the redox state of the T1Cu can be monitored ($\varepsilon_{595} = 6.3\ mM^{-1}\ cm^{-1}$), allowing the inter-Cu electron transfer to be studied. Laser photoexcitation of NADH using the 3rd harmonic of an Nd YAG laser ($\lambda_{exc} = 355$ nm) allows the rapid injection of electrons into the T1Cu of *Ax*NiR via direct reduction by solvated electrons or NADH· radicals, or via the mediator *N*-methyl nicotinamide. The initial fast reduction of T1Cu is not dependent on the NADH concentration ($k_{obs} = \sim 6000\ s^{-1}$), and results in the colourless Cu(I) state. The second observed rate constant shows a ~10-times slower re-oxidation of T1Cu of $\sim 370\ s^{-1}$ at 4 °C and pH 7.0 [30]. This second slower phase represents the redistribution of electrons between the two Cu sites based on the thermodynamic driving force, which is determined by the difference in reduction potentials of both copper centres (negligible in the absence of substrate: +255 mV for T1Cu and +244 for T2Cu) [41]. Complete redistribution towards the T2Cu site occurs only upon nitrite binding, indicating that substrate binding promotes electron transfer from T1Cu (Fig. 3A). The reduction potentials of the Cu centres affect both the thermodynamic equilibrium and the electron transfer kinetics. It has been proposed that substrate binding to the T2Cu induces a favourable shift in

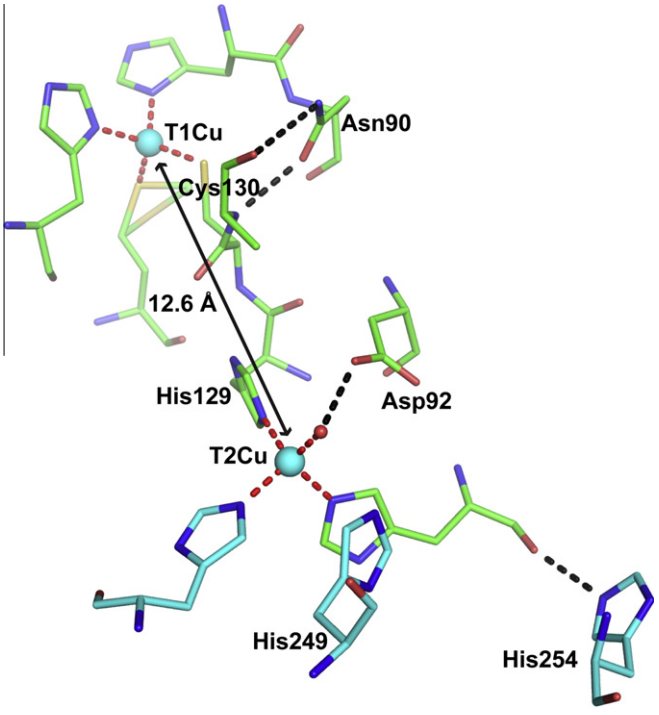

Fig. 2. Structure of the T1Cu and T2Cu sites in *Ax*NiR, the His–Cys electron transfer bridge, and the residues involved in putative proton transfer pathways. Residues originating from an adjacent monomer are shown in blue, Cu ions are shown as cyan spheres, and water molecules as smaller red spheres (pdb entry 1oe1) [37, 41].

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the reduction potential of the T2Cu [42, 43]. In fact, in the *Ax*NiR N90S mutant, where the T1Cu reduction potential is elevated by 60 mV, inter-Cu electron transfer is only observed in the presence of nitrite (*vide infra*), suggesting that the reduction potential of the T2Cu centre has increased by at least 60 mV upon binding of the substrate, allowing electron transfer to occur (Fig. 3B) [41]. It would be expected that a similar increase in the T2Cu reduction potential upon nitrite binding occurs in the native enzyme. Interestingly, a favourable shift in the T2Cu reduction potential would be expected to result in an accelerated electron transfer compared to the substrate-free reaction, however, the observed rates for electron transfer are lower in the presence of excess substrate at pH 7.0. This observation suggests that inter-Cu electron transfer in *Ax*NiR is controlled by other factors in addition to substrate-induced changes in the reduction potential.

3.2. Inter-copper electron transfer is coupled to proton transfer in AxNiR

The first indication for a coupled proton transfer and electron transfer event came from pulse radiolysis studies on the *Ax*NiR active site mutants D92A and H249A, which have a disrupted H-bonding network to the active site, resulting in drastically altered electron transfer rates [42]. Both Asp92 and His249 are suggested to provide a proton for the nitrite reduction [42, 44]. The temperature dependence of inter-Cu electron transfer in *Ax*NiR was studied in the presence of moderate nitrite concentrations (10 mM) between 4 and 40 °C [30]. A Marcus analysis of the data did not unequivocally demonstrate whether inter-Cu electron transfer is gated. Laser-photoexcitation in D_2O, however, resulted in an observed solvent kinetic isotope effect (SKIE), indicating that electron transfer is coupled to proton transfer (PCET). The detection of a SKIE in the absence of substrate ($\sim$1.33 ± 0.05) indicates that inter-Cu electron transfer is linked to a protonation step regardless of the chemical conversion of nitrite (Fig. 3D) [30]. The unusual substrate dependence of inter-Cu electron transfer in H_2O versus a 'normal' hyperbolic dependence in D_2O (Fig. 3C), resulting in an inverted SKIE at nitrite concentrations $\geqslant$0.1 mM, suggests the presence of various reaction routes that are differentially populated in H_2O and D_2O. Proton inventory experiments showed a linear relationship between k_{obs} for inter-Cu electron transfer in the substrate free enzyme upon increasing D_2O concentration, confirming that one solvent-exchangeable proton or one solvent molecule is involved in controlling the observed inter-Cu electron transfer rate irrespective of the chemical conversion of nitrite [30].

3.3. X-ray crystallography of AxNiR

The overall structure of CuNiRs consists of two eight-stranded β-barrel or cupredoxin folds for each monomer. The active site T2Cu is located at the subunit interface and is coordinated by His-residues from two adjacent monomers [37, 45]. Atomic resolution crystal structures of *Ax*NiR (1.04 Å), have identified the dual conformation of the two active site residues Asp92 and His249 [37], providing strong support for their critical role in substrate guidance, binding, and catalysis. Interestingly, inter-Cu electron transfer and conversion of nitrite has been observed during data collection in *Ax*NiR crystals *via* X-ray radiolysis. X-rays can reduce T1Cu directly but

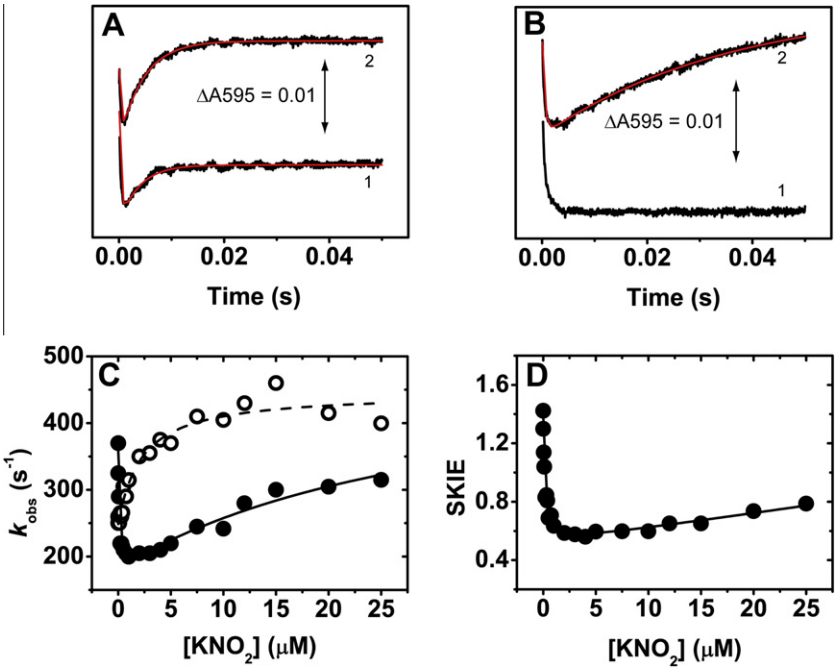

Fig. 3. (A) Absorbance changes at 595 nm observed in the laser flash photolysis instrument during reduction of wild-type *Ax*NiR and N90S (B) with NADH in the absence (trace 1) and in the presence of 10 mM nitrite (trace 2). Red lines represent fits to the data using a double exponential function. The initial fast decrease of the absorbance represents the reduction of the T1Cu by electrons generated upon excitation of NADH, the second phase represents electron transfer from T1Cu to T2Cu and is characterised by a slower recovery of the absorbance. In the absence of substrate electrons distribute between the two Cu sites based on the thermodynamic driving force. Complete redistribution towards the T2Cu site only occurs upon nitrite binding, indicating that substrate binding promotes electron transfer from T1Cu. Note that the lack of recovery of absorbance at 595 nm for N90S in the absence of substrate indicates that no electron transfer occurs unless substrate is present [41]. (C) Substrate dependence of electron transfer for wild-type *Ax*NiR observed in water (closed circles) and 95% D_2O (open circles). The solid line represents a fit of the data obtained in water to an equation reflecting two substrate-binding sites, and the dashed line represents a fit of the data obtained in D_2O to a hyperbolic one-site binding equation [30]. (D) Substrate dependence of the SKIE (solvent kinetic isotope effect, the ratio of the observed rate constants in water and D_2O). The solid line represents a fit to an equation reflecting two substrate-binding sites. The observed SKIE is indicative of a proton-coupled electron transfer process, and the detection of a SKIE in the absence of substrate indicates that inter-Cu electron transfer is linked to a protonation step regardless of the chemical conversion of nitrite [30].

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not T2Cu, which can only be reduced via internal electron transfer in the presence of substrate, resulting in a population of bound NO at T2Cu. These results provided evidence for substrate gating of inter-Cu electron transfer in *Ax*NiR by nitrite [46].

Two distinct proton channels that link the catalytic T2Cu to the surface of the protein have previously been identified in the atomic resolution crystal structures of *Ax*NiR [37, 47]. We have studied the functionality of the two putative proton channels, one involving Asn90 and the other involving His254 (Fig. 2). Crystal structures of both single mutants and the double mutant clearly show disrupted H-bonding networks in both channels as a result of the mutations [41, 47]. We found that the H254F mutation has no effect on the catalytic activity of *Ax*NiR, but that the N90S mutation results in ~70% decrease in enzyme activity. Laser-flash photolysis experiments show that in H254F electrons distribute between the two Cu sites based on the thermodynamic driving force similar to the wild-type enzyme. In N90S, where the T1Cu reduction potential is elevated by 60 mV, inter-Cu electron transfer is only observed in the presence of nitrite (Fig. 3B) [41]. From these results it is evident that the Asn90 channel is the main proton channel, although protons can still 'leak' to the active site, if this channel is structurally disrupted by mutagenesis. Crystallographic structures provide clear rationales for these observations including restoration of the proton delivery in N90S via a significant movement of the loop connecting T1Cu ligands Cys130 and His139 that occurs upon binding of nitrite.

In summary, these studies show that inter-copper electron transfer in *Ax*NiR can be conveniently studied using laser-flash photolysis under different conditions. Strong evidence is provided for a coupled proton and electron transfer event in *Ax*NiR that is chemically gated by its substrate nitrite. The tight coupling of proton and electron transfer in the presence of nitrite is designed in the structure to prevent the formation of a deactivated enzyme species with a prematurely reduced T2Cu.

4. Concluding remarks

Biological electron transfer reactions are complex and may be gated by protein motions and chemical steps. A combination of kinetic and thermodynamic studies, together with structural and biochemical data, are necessary to fully describe these reactions [2]. In this review, we illustrate the power of integrating the use of a range of experimental techniques, including X-ray crystallography, X-ray radiolysis, magnetic resonance and transient kinetics studies to dissect conformational and chemical gating of biological electron transfer reactions in the two model systems CPR and *Ax*NiR. Crystallographic data and PELDOR studies have revealed that conformational sampling appears to play a key role in inter-flavin electron transfer in CPR and may be induced by coenzyme binding itself. Laser-flash photolysis and X-ray radiolysis studies have provided strong evidence for a coupled proton and electron transfer in *Ax*NiR that is chemically gated by its small molecule substrate nitrite. Understanding the interplay between, and importance of, these control mechanisms is experimentally challenging. Integration of various experimental methods as discussed for CPR and *Ax*NiR provides a strategy for developing improved understanding of conformational and chemical gating mechanisms in other biological electron transfer reactions.

Acknowledgements

This work was supported by grants from the UK Biotechnology and Biological Sciences Research Council (BB/G005850/1, BB/G005869/1 and BB/G001383/1 and BB/D016290/1). N.S.S. is a BBSRC Professional Research Fellow and a Royal Society Wolfson Merit Award holder.

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