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Electrochemical and Kinetic Analysis of Electron-Transfer Reactions of *Chlorella* Nitrate Reductase†

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ABSTRACT: Assimilatory nitrate reductase (NR) from *Chlorella* is homotetrameric, each subunit containing FAD, heme, and Mo-pterin in a 1:1:1 stoichiometry. Measurements of NR activity and steady-state reduction of the heme component under conditions of NADH limitation or competitive inhibition by nitrite suggested intramolecular electron transfer between heme and Mo-pterin was a rate-limiting step and provided evidence that heme is an obligate intermediate in the transfer of electrons between FAD and Mo-pterin. In addition to the physiological substrates NADH and nitrate, various redox mediators undergo reactions with one or more of the prosthetic groups. These reactions are coupled by NR to NADH oxidation or nitrate reduction. To test whether intramolecular redox reactions of NR were rate-determining, rate constants for redox reactions between NR and several chemically diverse mediators were measured by cyclic voltammetry in the presence of NADH or nitrate. Reduction of ferrocenecarboxylic acid, dichlorophenolindophenol, and cytochrome *c* by NADH-reduced NR was coupled to reoxidation at a glassy carbon electrode (ferrocene and dichlorophenolindophenol) or at a bis(4-pyridyl) disulfide modified gold electrode (cytochrome *c*), yielding rate constants of 10.5×10^6, 1.7×10^6, and 2.7×10^6 M^{-1} s^{-1}, respectively, at pH 7. Kinetics were consistent with a second-order reaction, implying that intramolecular heme reduction by NADH and endogenous FAD was not limiting. In contrast, reduction of methyl viologen and diquat at a glassy carbon electrode, coupled to oxidation by NR and nitrate, yielded similar kinetics for the two dyes. In both cases, second-order kinetics were not obeyed, and reoxidation of dye-reduced Mo-pterin of NR by nitrate became limiting at low scan rates. Minimum estimates for NR reduction of 4.3×10^6 M^{-1} s^{-1} were obtained by extrapolation to infinite scan rate. These results suggest that reoxidation of reduced Mo-pterin by nitrate is rate-limiting for these partial activities of NR.

Intramolecular electron transfers between the prosthetic groups of assimilatory nitrate reductase (NR)[1] from *Chlorella* serve to conduct reducing equivalents from FAD, the site of NADH oxidation (Solomonson & Barber, 1990), via heme to the Mo-pterin[2] center, where nitrate is reduced to nitrite. The oxidation-reduction midpoint potentials of the three centers are consistent with essentially irreversible electron-transfer reactions in the physiological direction (Kay et al., 1988) since the electron affinities of the centers are well separated. In addition to NADH-dependent nitrate reduction, cytochrome *c* and a number of low molecular weight mediators can undergo redox reactions with one or more of the prosthetic groups. The partial activities which result from these reactions fall into two categories: NADH dehydrogenase activities, in which NADH-reduced NR may reduce cytochrome *c* (NADH:CR), DCPIP (NADH:DR), or ferricyanide (NADH:FR), and nitrate-reducing activities, in which reduced exogenous flavin (FH_2:NR), bromophenol blue (BPB:NR), or MV radical cation (MV:NR) act as electron donors (Solomonson & Barber, 1990). The relative rates of the partial activities indicate that NADH dehydrogenase activities are several times faster than the nitrate-reducing activities. Further evidence that rate limitation is associated with nitrate reduction was obtained from the observation that the heme is >80% reduced under conditions of optimal turnover (Kay & Barber, 1986). In the present study, we have investigated whether the NADH dehydrogenase and nitrate-reducing

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[1] Abbreviations: DCPIP, 2,6-dichlorophenolindophenol; DQ, diquat (6,7-dihydrodipyrido[1,2-*a*:2',1'-*c*]pyrazinediium); EDTA, ethylenediaminetetraacetic acid; FRC, ferrocenecarboxylic acid; G6P, D-glucose 6-phosphate; G6PDH, NADH:D-glucose dehydrogenase (EC 1.1.1.49); MOPS, 3-(*N*-morpholino)propanesulfonic acid; MV, methyl viologen (1,1'-dimethyl-4,4'-bipyridinium dichloride); MV:NR, reduced methyl viologen:nitrate reductase; NADH:CR, NADH:cytochrome *c* reductase; NADH:FRCR, NADH:ferrocenecarboxylic acid reductase; NR, nitrate reductase; LSV, linear sweep voltammetry; NHE, normal hydrogen electrode; GCE, glassy carbon electrode.
[2] The term "Mo-pterin" refers to the complex of Mo, molybdopterin, and associated protein ligands to Mo.

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partial activities of NR are rate-limited by intramolecular or intermolecular electron transfers. A simple kinetic model for heme behavior is derived and tested using linear sweep voltammetry to couple partial reactions to electrochemical measurements.

Direct electron transfer between redox proteins and electrodes is possible in only a few cases (Armstrong et al., 1986; Frew & Hill, 1988). One example is cytochrome *c*, which exhibits quasi-reversible electrochemistry at a gold electrode modified with bis(4-pyridyl) disulfide, in the absence of dissolved mediator (Taniguchi et al., 1982). In contrast to proteins, several of the low molecular weight redox mediators which react with NR also exhibit well-behaved electrochemistry at suitable electrode surfaces. It is therefore possible to couple many of the partial enzyme activities of NR to electrochemical detection and derive information on the reaction rate constant between mediator and protein. We have applied this technique to determine rate constants for the oxidation and reduction of cytochrome *c* and other mediators by NR which, together with the results of steady-state kinetic studies, has allowed us to reach tentative conclusions concerning the rate-limiting electron-transfer reactions of NR.

Experimental Procedures

Enzyme Preparation. Nitrate reductase was isolated from *Chlorella vulgaris* as previously described (Howard & Solomonson, 1981), with an additional purification by HPLC using a TSK4000 column. The enzyme exhibited an activity of greater than 80 units/mg of protein and an A_{280}/A_{413} ratio of less than 1.8. The NR heme concentration was determined using an extinction coefficient of 117 mM^{-1} cm^{-1} (Solomonson et al., 1984). Reduced horse heart cytochrome *c* (type VI, Sigma) was prepared by addition of 3 mM sodium ascorbate to a 2 mM cytochrome *c* solution in 50 mM potassium phosphate buffer, pH 7.0, followed by Sephadex G-25 gel filtration to remove excess ascorbate. The absence of ascorbate was verified by stoichiometric titration of reduced cytochrome *c* with potassium ferricyanide solution. Cytochrome *c* oxidation was monitored spectroscopically at 550 nm, and immediate oxidation of cytochrome *c* by small aliquots of ferricyanide indicated that excess ascorbate was absent.

Steady-State Heme Reduction. The extent of NR heme reduction during steady-state turnover was determined at 423 nm in a Shimadzu UV260 spectrophotometer (Columbia, MD) equipped with a cuvette stirrer. In one set of experiments, the rate of NADH production was varied using a coupled assay consisting of D-glucose 6-phosphate (2 mM), NAD^+ (0.1 mM), KNO_3 (1 mM), and varying activities (0.5–6.0 units/mL, determined spectrophotometrically under identical assay conditions) of glucose-6-phosphate dehydrogenase (Sigma type XXIV from *Leuconostoc mesenteroides*). Samples of NR (0.5–2.0 μM heme) were added to buffer (224 mM MOPS Ultrol grade and 0.1 mM EDTA, pH 8.0) containing NAD^+. Glucose-6-phosphate dehydrogenase was added, resulting in total reduction of NR heme (confirmed by the addition of sodium dithionite which produced no further spectral change). KNO_3 was added, and a fraction of the NR heme became oxidized during catalysis. Once the nitrate was depleted, the heme again became reduced. In a second series of experiments, the rate of NR turnover was varied by the addition of KNO_2, a product inhibitor (Howard & Solomonson, 1981). The concentrations of nitrite necessary to inhibit NR by up to 90% were calculated from

$$K_{m,app} = K_m(1 + [NO_2^-]/K_I)$$

$$v/V_m = [NO_3^-]/(K_{m,app} + [NO_3^-])$$

These equations treat nitrite inhibition as competitive with respect to nitrite and are applicable to NR when NADH (1 mM, K_m = 4 μM) is saturating (Howard & Solomonson, 1982). Values appropriate to the present assay conditions of 60 and 350 μM were used for $K_{m,app}$ and K_I, respectively (Kay & Barber, 1989). The extent of inhibition was measured by determining rates of NADH oxidation at 340 nm. Samples of NR in MOPS buffer were reduced by addition of 1 mM NADH in the presence of varying concentrations of nitrite. Enzyme turnover was initiated by addition of 1 mM KNO_3, and heme reduction was monitored at 423 nm.

Cyclic Voltammetry. Cyclic voltammetry experiments were performed at 25 °C using a BAS CV-1B potentiostat (BAS Inc., West Lafayette, IN). A glass cell of 0.7-mL internal volume was maintained anaerobic by flushing with O_2-free Ar. A standard three-electrode apparatus was used, comprising either a 1.6-mm-diameter gold disk or a 3.2-mm-diameter planar GCE as the working electrode (BAS Inc., West Lafayette, IN), a gold auxiliary electrode, and a Ag/AgCl reference electrode (Microelectrodes Inc., Londonderry, NH). The reference electrode was calibrated before each experiment using a saturated quinhydrone solution (Eastman Kodak, Rochester, NY).

Cyclic voltammetry of cytochrome *c* was achieved at a modified gold electrode. Prior to each experiment, the gold working electrode was polished with a 0.4-μm alumina/water slurry and cleaned in a sonicating water bath. The gold working electrode was modified according to the method of Taniguchi et al. (1982) to permit direct electrochemistry of horse heart cytochrome *c* (type VI). The freshly cleaned electrode surface was immersed in a 1 mM solution of bis-(4-pyridyl) disulfide in deionized water for 5 min and then rinsed thoroughly with deionized water and used without delay. Voltammetric responses of FRC, DCPIP, MV, and DQ were obtained at unmodified glassy carbon electrodes (BAS Inc., West Lafayette, IN).

Data Analysis. The enhancement in anodic or cathodic currents observed in the presence of NR and substrate was analyzed as described by Nicholson and Shain (1964). Values of i_k/i_d (the ratio of catalytic to diffusion current) were determined experimentally and converted to values of k_f/a, where $a = nFv/RT$, by computer interpolation from the values presented by Nicholson and Shain (1964) for the case vii. Primary plots of k_f/a versus reciprocal scan rate were constructed to derive values of k_f (s^{-1}) from the gradient at each NR concentration used. Secondary replots of k_f versus [NR] were used to derive the second-order rate constants for reaction of NR with mediator.

Results

Spectroscopic measurements of the degree of NR heme reduction during steady-state nitrate reduction have been reported previously under conditions of high and low ionic strength (Kay & Barber, 1986). These results were consistent with rate-limiting electron transfer from heme to Mo–pterin centers. In the present work, these observations have been extended by the measurement of heme reduction when the rate of enzyme catalysis was decreased either by limiting NADH supply or by the product inhibitor nitrite. Figure 1A shows the response of NR heme in the presence of NO_3^- and a G6PDH-coupled assay system (Cottingham & Moore, 1983; Kay & Barber, 1989) which reduces NAD^+. The activity of G6PDH added to the assay was determined under identical assay conditions from the rate of NADH production in the

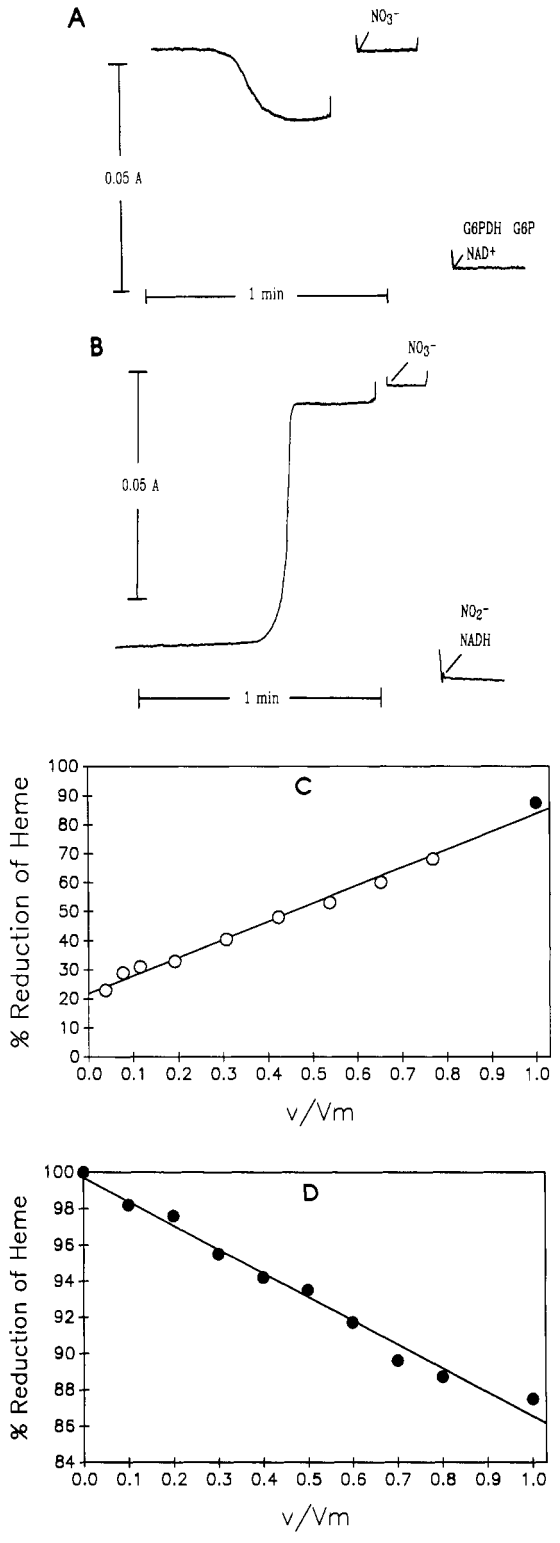


FIGURE 1: Reduction of NR heme during steady-state catalysis. (A) The degree of reduction of NR heme (0.56 μM) was monitored at 423 nm in a stirred cuvette as described under Experimental Procedures. G6PDH (2 units) was added to initiate NAD^+ and NR reduction. The G6PDH activity corresponded to 78% of the NADH:NR activity measured under identical conditions. (B) Heme reduction under conditions of nitrite inhibition. NR (0.72 μM heme) in the presence of 8.4 mM KNO_2 was reduced by addition of NADH, and turnover was initiated by addition of 1 mM KNO_3. The extent of inhibition by nitrite was 60%. (C) Steady-state heme reduction related to rates of NR turnover. Heme reduction was determined as in (A), with varying G6PDH activity to regulate NADH production. Rates are expressed as a proportion of the rate obtained in the presence of 1 mM NADH. (D) Relationship between rate of turnover (v/V_m) and heme reduction. Rate of NR turnover was determined by the nitrite concentration.

absence of NR. The optimum NADH:NR activity was determined at saturating substrate concentrations (1 mM NADH, 1 mM NO_3^-) to establish the amount of G6PDH activity required. The amount of G6PDH activity added to the assay was adjusted to correspond to a proportion of the optimum NADH:NR activity. In Figure 1A, this proportion was 0.78. Addition of NO_3^- to NADH-reduced NR resulted in oxidation of 30% of the heme. The degree of reduction remained essentially constant for several seconds until nitrate (1 mM initial concentration) became exhausted, and the heme became fully reduced again, as verified by dithionite reduction.

For this assay to be a valid measure of heme reduction under steady-state conditions, two conditions have to be satisfied. First, the level of heme reduction during nitrate reduction must remain essentially constant for several seconds to allow measurement. This degree of stability was observed, and was presumably due to the relative kinetic constants of the assay system. Product inhibition of NR by NAD^+ (K_I = 1.6 mM) is weak relative to the K_m for NADH (4 μM; Howard & Solomonson, 1981), and product inhibition by NO_2^- (K_I = 360 μM) is weak relative to the K_m for NO_3^- (80 μM; Kay & Barber, 1989). NR activity will therefore be only slowly inhibited by the buildup of NO_2^- during the assay, while the steady-state concentrations of NADH and NAD^+ which will be established will be low for NADH (since the K_m for NR is 4 μM), while the NAD^+ concentration (close to 100 μM) will cause only small inhibition of NR, and will stay close to the K_m for G6PDH (90 μM). The other product of the G6PDH-catalyzed reaction (D-glucose δ-lactone 6-phosphate) was found to be a weak inhibitor at the saturating (2 mM) concentrations of G6P used (results not shown). These relative kinetic constants result in a measurably long steady-state heme reduction following addition of G6PDH. Second, the rate of nitrate reduction by NR is assumed to be determined by the activity of G6PDH added. The kinetic constants given above indicate that this would be true; however, this was also confirmed by colorimetric determination of nitrite production in the coupled assay (results not shown).

The effect of heme reduction of inhibiting NR by NO_2^- product inhibition at saturating NADH concentrations is shown in Figure 1B. The NO_2^- concentration assay necessary for a given degree of inhibition was calculated from the published kinetic constants (see Experimental Procedures) and confirmed by the initial rate assay. Inhibition of 60% of the NADH:NR activity resulted in a steady-state level of heme reduction of 94%. Following NADH exhaustion, it was consistently observed that the heme did not fully reoxidize. Approximately 20% of the heme remained reduced under the assay conditions of Figure 1A,B when NO_3^- was present in excess. This is presumably due to the presence of demolybdonitrate reductase, which has previously been reported to constitute 15–20% of the NR purified from *Chlorella*, determined by colorimetric analysis of Mo content (Howard & Solomonson, 1981). The residual reduced heme was reoxidized following addition of ferricyanide (results not shown).

A series of heme reduction measurements, obtained at various rates of NR turnover, are shown in Figure 1C,D. In both the NADH-limited case (Figure 1C) and NO_2^--inhibited cases (Figure 1D), a linear relationship was observed between the rate of NR activity, expressed as a fraction of the optimum activity (v/V_m), and the observed degree of steady-state heme reduction. The intercept of Figure 1C represents demolybdo enzyme as previously mentioned. These linear relationships are readily described in terms of a simple two-step model consisting of one composite rate constant (k_{red}) for heme re-

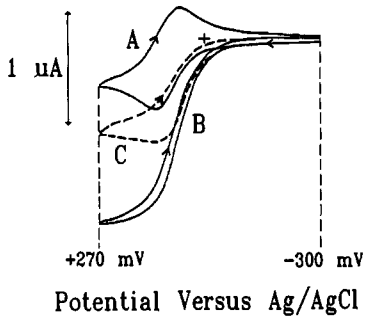


FIGURE 2: Coupled electrochemical and enzymatic reactions of cytochrome *c*. A typical voltammogram of reduced cytochrome *c* (0.4 mM total concentration) at a bis(4-pyridyl) disulfide modified gold electrode is shown (trace A). The assay medium was 50 mM potassium phosphate, 0.1 mM EDTA, and 2 mM NADH, pH 7. Working electrode diameter was 1.6 mm, and scan rate was 5 mV/s. (Trace B) As for trace A, but with the addition of 75 nM NR. (Trace C) As for trace A, but with the addition of 30 nM NR.

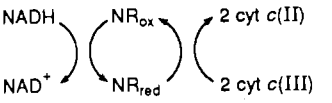
duction by NADH and a similar composite rate constant (k_{ox}) for heme oxidation by NO_3^-:

$$\text{NADH} \xrightarrow{k_{red}} \text{heme} \xrightarrow{k_{ox}} NO_3^-$$

The two rate constants can be varied either by limiting NADH supply (Figure 1C) or by NO_2^- inhibition (Figure 1D). The rate of NO_3^- reduction (v) is given by $k_{red}k_{ox}/(k_{red} + k_{ox})$, while the degree of heme reduction is given by $k_{red}/(k_{red} + k_{ox})$. A linear relationship is predicted between heme reduction and the rate of turnover. The slopes of Figure 1C,D indicate that $k_{red} > k_{ox}$. The V_m for NADH:NR activity is 9.1 μmol of $2e^-$ min^{-1} (nmol of NR heme)$^{-1}$. Solving this scheme using the slopes of Figure 1C,D yielded values for k_{red} and k_{ox} of 45.0 and 11.4 μmol of $2e^-$ min^{-1} (nmol of NR heme)$^{-1}$, respectively. These values are similar to previously reported values, determined under similar assay conditions, for the V_m values of the partial activities NADH:CR [39.9 μmol of $2e^-$ min^{-1} (nmol of NR heme)$^{-1}$ and MV:NR [14.5 μmol of $2e^-$ min^{-1} (nmol of NR heme)$^{-1}$] which are believed to correspond to the intramolecular electron-transfer processes given by k_{red} and k_{ox}, respectively (Kay & Barber, 1986). This suggested that intramolecular processes limited the V_m values observed for these procedures.

To determine whether the V_m values for the partial activities NADH:CR and MV:NR were rate-limited by intramolecular or intermolecular electron transfers, linear sweep voltammetric analysis was utilized. In this technique, the oxidation or reduction of the various mediators used to measure the partial enzyme activities of NR is coupled to electrochemical regeneration of the appropriate species at a suitable electrode surface using either NADH as the reductant or NO_3^- as the oxidant, respectively. By analysis of the enhancement in electrode current observed at various scan rates, which results from the homogeneous enzyme reaction, it is possible to determine whether regeneration of the appropriate redox form of NR is rate-limiting. If it is not, then a value can be obtained for the second-order rate constant for reaction between NR and mediator (Nicholson & Shain, 1964). At a bis(4-pyridyl disulfide modified gold electrode, reduced horse heart cytochrome *c* exhibited a quasi-reversible cyclic voltammogram (trace A, Figure 2) as previously reported (Taniguchi et al., 1982). Anodic peak currents increased linearly as a function of the square root of scan rate, yielding a cytochrome *c* diffusion coefficient (D) of 1.2×10^{-6} cm^2 s^{-1}, close to previously reported values (Eherenberg, 1957; Taniguchi et al., 1982). Addition of either NADH (5 mM) or NR (0.2 μM heme) alone had no effect on the current–potential curve. However,

Scheme I



the presence of both NADH and NR resulted in an enhanced anodic current and decreased cathodic current (trace B, Figure 2), consistent with catalytic regeneration of reduced cytochrome *c* by NADH-reduced NR in the diffusion layer of the electrode, according to Scheme I.

At saturating NADH concentrations, the perturbation of cytochrome *c* voltammetry observed in the presence of NR may be analyzed according to Scheme vii of Nicholson and Shain (1964):

$$R \rightarrow O + e^- \quad (1)$$

$$O + Z \xrightarrow{k_f} R \quad (2)$$

where reaction 1 is the heterogeneous oxidation of ferrocytochrome *c* (R) to ferricytochrome *c* (O) at the electrode and reaction 2 is a second-order homogeneous reduction of ferricytochrome *c* by NADH-reduced NR (Z), with rate constant k_f such that $k = k_f[Z]$. For this scheme to be applicable, reaction 1 must be rapid compared to reaction 2 and reversible. In addition, the immediate donor to cytochrome *c*, which is the heme of NR, must be rapidly reduced by NADH so that the heme is always reduced. Saturating concentrations (5 mM, K_m = 4 μM; Howard & Solomonson, 1982) of NADH were present in the reaction. The analysis of Nicholson and Shain (1964) (see Experimental Procedures) was applied to voltammograms obtained in the presence of a variety of NR concentrations and for a range of scan rates. Plots of k_f/a against reciprocal scan rate obtained at pH 7 and 25 °C were linear within the range tested (5–130 mV/s). The slope obtained at each NR concentration is equal to k_fRT/nF. The replot of k_f versus NR concentration was linear over the NR concentration range of 0–180 nM (results not shown) and yielded a second-order rate constant for reduction of cytochrome *c* by reduced NR of 2.7×10^6 M^{-1} s^{-1}. The consistency of the observed kinetics with eq 1 and 2 suggested that intramolecular NR heme reduction by NADH and endogenous FAD was not rate-limiting in cytochrome *c* reduction.

A similar analysis was repeated for FRC and DCPIP, which exhibited quasi-reversible electrochemistry at a GCE and are also readily reduced by NR. Plots of k_f/a against reciprocal scan rate for FRC (0.4 mM), obtained at 7 and 25 °C, were linear within the range tested (5–100 mV/s). In addition to the enzymatic reduction of ferrocene by NR, a slower nonenzymatic reduction of ferrocene by NADH was observed. Plots of k_f/a versus $1/v$ and of k_f versus [NADH] (2–10 mM) were linear (results not shown) and were consistent with a reaction rate constant of 9.4×10^4 M^{-1} s^{-1} for the nonenzymatic reaction. The nonenzymatic contribution is observed as the intercept on the k_f versus [NR] (30–150 nM) plot, contributing 0.2 s^{-1} to k_f at an NADH concentration of 5 mM, in good agreement with data obtained from the corresponding k_f/a versus $1/v$ plot (results not shown). The linearity of this plot indicated that re-reduction of NR by NADH does not limit NADH:FRCR activity. The derived rate constant for NADH:FRCR was 10.5×10^6 M^{-1} s^{-1}.

Results obtained with DCPIP (0.4 mM), which interacts with NR heme (Kay & Barber, 1986), in the presence of NADH (2 mM) yielded linear plots for both k_f/a versus reciprocal scan rate (5–200 mV/s) and k_f versus [NR] (50–450 nM) and were also consistent with rapid regeneration

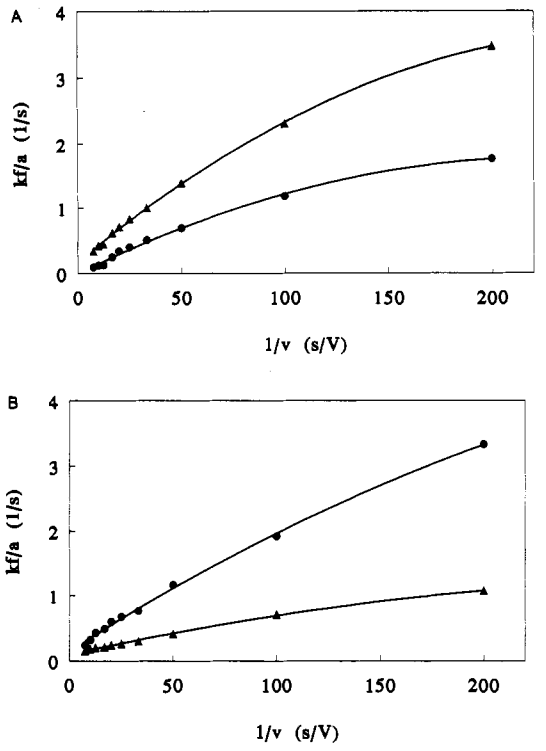


FIGURE 3: Oxidation of reduced MV and DQ by NR. (A) Assay conditions were 50 mM potassium phosphate and 0.1 mM EDTA, pH 7, containing 0.4 mM DQ and KNO_3 (5 mM). (●) 111 nM NR; (▲) 222 nM NR. (B) As for (A), but with 0.4 mM MV replacing DQ.

of the reduced species of NR. The derived rate constant for NADH:DR was 1.7×10^6 M^{-1} s^{-1}.

The one-electron-reduced forms of MV and DQ very rapidly reduce NR and can be used to support NO_3^- reduction, designated MV:NR and DQ:NR activities, respectively. These compounds were readily reduced at a glassy-carbon electrode and exhibited two separate waves of reduction, corresponding to two $n = 1$ reductions, with production of the radical species at −450 mV versus NHE. By restriction of the potential to >−600 mV, the second reductive process was avoided. Neither the kinetics of MV nor the kinetics of DQ were consistent with Scheme I, since plots of k_f/a versus $1/v$ were nonlinear (Figure 3). The curvature of these plots is consistent with rate-limiting regeneration of oxidized NR Mo–pterin by NO_3^-. Minimum estimates of the rate constants for the reactions of these reduced mediators with NR were obtained by extrapolation to infinite scan rates. Both gave estimates of $>4.3 \times 10^6$ M^{-1} s^{-1}.

The individual rate constants obtained by LSV for the reactions of the various mediators with NR are summarized in Table I.

Table I: Summary of Rate Constants for Reaction of Nitrate Reductase with Mediators Determined by LSV[a]

reaction	NR substrate	k (M^{-1} s^{-1})
NR(red) + cyt c(ox) → NR(ox) + cyt c(red)	NADH	2.7×10^6
NR(red) + FRC(ox) → NR(ox) + FRC(red)	NADH	10.5×10^6
NR(red) + DCPIP(ox) → NR(ox) + DCPIP(red)	NADH	1.7×10^6
NR(ox) + MV(red) → NR(red) + MV(ox)	nitrate	$>4.3 \times 10^6$
NR(ox) + DQ(red) → NR(red) + DQ(ox)	nitrate	$>4.3 \times 10^6$

[a] Rate constants were derived from plots of k_f/a versus $1/v$ for the various mediators. The values presented for MV and DQ represent minimum values and were derived from plots of k_f/a versus $1/v$ by extrapolation to infinite scan rate.

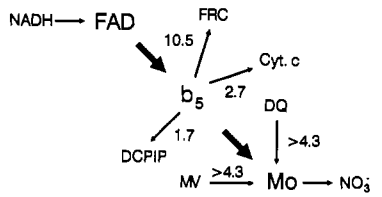


FIGURE 4: Schematic diagram of the various electron-transfer reactions of NR. Intramolecular electron-transfer reactions between the FAD, heme, and Mo–pterin prosthetic groups are shown in bold arrows. Sites of interaction of the physiological reducing (NADH) and oxidizing (NO_3^-) substrates are also shown as are the sites of interaction of the various mediators and the second-order rate constants ($\times 10^6$ M^{-1} s^{-1}) determined in this study.

Discussion

The steady-state determinations of heme reduction presented above suggested a simple model of heme behavior in terms of two gross rate constants, which would in fact be complicated functions of the elementary rate processes comprising a detailed catalytic cycle for NR. The simple model was consistent with the heme steady-state results, in that the linear relationship between heme reduction and catalytic rate was predicted under conditions of nitrite inhibition and NADH limitation. These results support the often assumed role of heme as an obligate intermediate in electron transfer between FAD and Mo–pterin. The unidirectional flow of electrons during the heme reduction and oxidation processes is supported by the large increases in midpoint potential from NADH (−340 mV) to heme (−164 mV) to NO_3^-/NO_2^- (+420 mV). In addition to explaining the heme steady-state results, the model predicted values for the two rate constants from the NADH:NR V_m value. These values were close to the V_m values for the partial enzymatic activities which involve the relevant prosthetic groups of the enzyme; NADH:CR involves only the FAD and heme centers, while MV:NR requires Mo–pterin. We therefore wished to determine whether these partial enzyme activities were rate-limited by processes intramolecular to NR as predicted by the simple model or were limited by the intermolecular reactions with the mediators, which would invalidate the model by showing that intramolecular processes were faster than predicted.

Cyclic voltammetry has been used in a number of studies to characterize reactions between mediators and redox enzymes [e.g., see Hill and Walton (1982), Cass et al. (1985), and Coury et al. (1990)]. The approach has advantages over conventional kinetic methods and also limitations. Mediators which are unstable in one redox form can be used because the unstable species is rapidly and reversibly generated only in the diffusion layer of the electrode. Mediators which react with dissolved oxygen may be used, since the voltammetry is performed anaerobically. No spectral signal is necessary to follow changes in mediator redox state. However, the equations which describe the current–time curve are difficult to solve (Nicholson & Shain, 1964), and one assumption made is that the diffusion coefficients for mediator and reactant are identical, which is an approximation when applied to enzyme/mediator reactions. In the present study, the questions addressed were the following: Do the coupled mediator reactions qualitatively follow the kinetics of Scheme vii of Nicholson and Shain (1964), and, if so, what is the second-order rate constant for the homogeneous reaction?

The various electron-transfer reactions of NR and their second-order rate constants examined in this study are shown in Figure 4. LSV yielded second-order rate constants at pH

7.0 of 10.5×10^6, 1.7×10^6, and 2.7×10^6 M^{-1} s^{-1} for the reduction by NADH-reduced NR of FRC, DCPIP, and cytochrome *c*, respectively. Cass et al. (1985) have reported a comparable value of 5×10^6 M^{-1} s^{-1} for the reduction of cytochrome *c* by L-lactate-reduced flavocytochrome b_2. The kinetics of the NR reaction were fully consistent with a simple second-order reaction. Cytochrome *c* and DCPIP are reduced by NR heme, since reduction is sensitive to limited proteolytic cleavage of the enzyme into FAD and heme/Mo–pterin-containing fragments. Mo–pterin is not required since substitution of W for Mo, which abolishes NADH:NR activity, has no effect on these NADH dehydrogenase activities (Solomonson et al., 1984). Reduction of ferrocenecarboxylic acid appeared to be sensitive to limited digestion by V8 protease, since digested NR samples no longer perturbed the FRC voltammogram (results not shown), suggesting FRC is also reduced by NR heme. The second-order reaction kinetics for the NADH dehydrogenase activities indicated that intramolecular heme reduction by NADH and FAD was not rate-limiting in the regeneration of reduced NR. Within the diffusion layer of the electrode, the concentration of reduced NR heme remained constant and was not depleted by reaction with the mediator at scan rates as low as 5 mV/s. The medium in which the LSV was performed was close to the optimum ionic strength for NR activity, and results in comparatively weak binding of cytochrome *c* to NR (K_m = 22 μM; Kay & Barber, 1986). We conclude that NADH:CR activity is not limited by the internal electron-transfer rate constants for reduction of NR heme by NADH. The correspondence of NADH:CR V_m values and the calculated value for k_{red} probably result from the simplistic nature of this model, and further work to elucidate the individual steps in heme reduction is required.

In contrast, MV and DQ exhibited identical kinetics for NR-catalyzed nitrate reduction. In both cases, second-order kinetics were not observed, and reoxidation of dye-reduced Mo–pterin by nitrate became limiting at low scan rates, as shown by the curved k_f/a versus $1/v$ plots. This indicated that the catalytic rates of these partial activities are limited by reactions of nitrate at the Mo–pterin center. Minimum estimates for Mo–pterin reduction of 4.3×10^6 M^{-1} s^{-1} were obtained by extrapolation to infinite scan rate, but the true rates may be significantly faster due to the large potential difference between the $MV^{2+}/MV^{\bullet+}$ (−411 mV) and Mo(VI)/Mo(IV) (−5 mV) redox couples.

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