Spinach Nitrate Reductase
Effects of Ionic Strength and pH on the Full and Partial Enzyme Activities

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ABSTRACT

Initial velocity studies of immunopurified spinach nitrate reductase have been performed under conditions of controlled ionic strength and pH and have been in the absence of chloride ions. Increased ionic strength stimulated NADH:ferricyanide reductase and reduced flavin:nitrate reductase activities and inhibited NADH:nitrate reductase, NADH:cytochrome c reductase and reduced methyl viologen:nitrate reductase activities. NADH: dichlorophenolindophenol reductase activity was unaffected by changes in ionic strength. All of the partial activities, expressed in terms of micromole 2 electron transferred per minute per nanomole heme, were faster than the overall full, NADH:nitrate reductase activity indicating that none of the partial activities included the rate limiting step in electron transfer from NADH to nitrate. The pH optimum for NADH:nitrate reductase activity was determined to be 7 while values for the various partial activities ranged from 6.5 to 7.5. Chlorate, bromate, and iodate were determined to be alternate electron acceptors for the reduced enzyme. These results indicate that unlike the enzyme from Chlorella vulgaris, intramolecular electron transfer between reduced heme and Mo is not rate limiting for spinach nitrate reductase.

In addition to catalyzing the physiological reaction the enzyme exhibits a number of partial activities which utilize one or more of the enzyme cofactors (8). These partial activities have been divided into two classes referred to as either diaphorase or nitrate-reducing activities, respectively, corresponding to whether they utilize NADH as the electron donor or utilize nitrate as the terminal electron acceptor. Diaphorase activities require a functional flavin center comprising the NADH-binding site, FAD and an essential sulfhydryl group, for catalytic activity whereas nitrate-reducing activities can utilize artificial electron donors and require an active Mo center. Some activities, such as those involving electron transfer to Cyt c or DCIP or from exogenous FADH2 also require the heme center. The diaphorase activities comprise NADH:FR, NADH:CR, and NADH:DR activities whereas the nitrate-reducing activities comprise reduced flavin:nitrate reductase (FH2:NR) and MV:NR activities.

Limited proteolysis studies have resolved the cofactor requirements for the various partial activities (12). Proteolytic cleavage and separation of the two fragments formed following treatment of the spinach enzyme with corn inactivator or V8 protease has shown that the 'small' fragment is required for all diaphorase activities and contains both FAD and the NADH-binding site whereas the 'large' fragment contains heme and Mo and retains FH2:NR and MV:NR activities.

Previous work using the enzyme isolated from the green algae Chlorella vulgaris (10), has demonstrated that ionic strength and pH, differentially influence the rates of the full and partial activities of NR and that the rate-limiting step in turnover of the Chlorella enzyme involves electron transfer from the b-type Cyt to Mo-pterin. We have extended these studies to the spinach enzyme and have demonstrated that this intramolecular electron transfer rate limitation is not conserved in spinach nitrate reductase.

MATERIALS AND METHODS

Assimilatory NADH:NR was isolated from freshly harvested spinach leaves (Spinacea oleracea, L.) using immunochromatography as previously described (5) and stored in 50% glycerol. Enzyme concentrations were determined using \( E_{1%} = 117 \text{ mm}^{-1} \cdot \text{cm}^{-1} \). Enzyme activities, expressed as initial rates for the transfer of either one or two reducing equivalents per nmol heme, were determined at 25°C as previously de-
The effects of ionic strength on the full and partial activities catalyzed by spinach nitrate reductase at pH 7 and 25°C in MES buffer, containing 0.1 mM EDTA and 5 μM FAD, are shown in Table I. All enzyme activities were assayed at pH 7, the pH optimum for the physiological NADH:NR activity, to facilitate comparison of the effects of increasing ionic strength on the various partial activities at constant pH. Previous data obtained for the full, NADH:NR activity (1), showed a biphasic response to ionic strength. Lineweaver-Burke plots for NADH:NR activity were linear at all substrate concentrations examined and indicated an initial increase in activity with increasing ionic strength which reached a maximum rate, corresponding to 9 μmol 2e/min/nmol heme, at approximately μ = 50 mM but which decreased at higher ionic strength. Under conditions of optimal ionic strength, (μ = 50 mM), Kₘ's for NADH and NO₃⁻ were determined to be 7 and 13 μM, respectively. At the highest ionic strength examined (μ = 300 mM), NADH:NR activity had decreased to 52% (4.7 μmol 2e/min/nmol heme) of maximum. Changing the nature of the buffer from MES to MOPS, to ensure adequate buffering capacity at low ionic strength, had no significant effect on the rates of either the full or partial activities.

Increasing the ionic strength from 10 to 50 mM, resulted in an initial 20% stimulation of NADH:FR activity, corresponding to rates of 224 and 269 μmol 2e/min/nmol heme, respectively, the rate remaining constant at higher ionic strengths. However, a more substantial change in the Kₘ for Fe(CN)₆⁴⁻ was observed, which increased by approximately 67% from 21 μM (μ = 50 mM) to a maximum of 35 μM (μ = 200 mM).

Ionic strength had only a limited effect on NADH:CR activity, decreasing Vₘₐₓ by 8%, from 83 to 76 μmol 2e/min/nmol heme at μ = 50 mM and 200 mM, respectively. In contrast, increasing the ionic strength from 50 mM to 200 mM resulted in an approximate six-fold increase, from 6 to 29 μM, in Kₘ for Cyt c.

Elevated ionic strength had little influence on either the Vₘₐₓ for NADH:DR activity or the Kₘ for DCPIP. Rates and Kₘ's remained effectively unchanged, corresponding to 132 and 138 μmol 2e/min/nmol heme and 53 and 56 μM, respectively, on increasing the ionic strength from 50 to 200 mM.

Increased ionic strength was also observed to differentially influence the partial activities involving the artificial electron donors, FADH₂ and MV⁺. At pH 7 and in the presence of near-saturating concentrations of nitrate, increased ionic strength was found to stimulate FH₂:NR activity which reached a maximum rate of 20 μmol 2e/min/nmol heme at approximately μ = 200 mM, corresponding to an apparent 1.7-fold increase in activity over the rate obtained at μ = 50 mM, while the Kₘ for FADH₂ remained unchanged at 56 μM.

In contrast, elevated ionic strength was found to inhibit MV:NR activity. At low ionic strengths (μ < 50 mM), MV:NR activity was initially enhanced by increased ionic strength, reaching a maximum rate of 32 μmol 2e/min/nmol heme at μ = 50 mM. However, higher ionic strengths resulted in substantial inhibition of activity, which decreased to approximately 56% (18 μmol 2e/min/nmol) of the maximal value at μ = 200 mM.

### Alternative Electron Acceptors for Spinach Nitrate Reductase

In addition to utilizing nitrate as the electron acceptor, spinach nitrate reductase was capable of transferring reducing equivalents to several artificial electron acceptors including FADH₂ and DCPIP. The effects of ionic strength on the full and partial activities catalyzed by spinach nitrate reductase at pH 7 and 25°C in MES buffer, containing 0.1 mM EDTA and 5 μM FAD, are shown in Table I. All enzyme activities were assayed at pH 7, the pH optimum for the physiological NADH:NR activity, to facilitate comparison of the effects of increasing ionic strength on the various partial activities at constant pH. Previous data obtained for the full, NADH:NR activity (1), showed a biphasic response to ionic strength. Lineweaver-Burke plots for NADH:NR activity were linear at all substrate concentrations examined and indicated an initial increase in activity with increasing ionic strength which reached a maximum rate, corresponding to 9 μmol 2e/min/nmol heme, at approximately μ = 50 mM but which decreased at higher ionic strength. Under conditions of optimal ionic strength, (μ = 50 mM), Kₘ's for NADH and NO₃⁻ were determined to be 7 and 13 μM, respectively. At the highest ionic strength examined (μ = 300 mM), NADH:NR activity had decreased to 52% (4.7 μmol 2e/min/nmol heme) of maximum. Changing the nature of the buffer from MES to MOPS, to ensure adequate buffering capacity at low ionic strength, had no significant effect on the rates of either the full or partial activities.

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### Table I. Influence of Ionic Strength on the Full and Partial Activities of Spinach Nitrate Reductase

All activities were determined at 25°C in either MOPS (μ ≤ 50 mM) or MES (μ ≥ 50 mM) buffer (pH 7.0), conditions under which the full, NADH:NR activity was maximal.

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<td></td>
<td>% maximal activity</td>
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<tr>
<td>10</td>
<td>85</td>
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equivalents to a variety of halogenates including chlorate, bromate and iodate. At pH 7 (µ = 50 mM) and using near-saturating concentrations of NADH, substitution of chloride for nitrate resulted in equivalent maximal activity (9 µmol 2e/min/nmol heme) although the $K_m$ for chloride was 17 times higher than that for $NO_3^-$ (Table II). Use of bromate resulted in a 67% stimulation of activity with a corresponding 57-fold increase in $K_m$. In contrast, substitution of iodate for nitrate resulted in a 33% reduction in maximal activity and a corresponding 800-fold increase in $K_m$.

**Effects of pH on the Catalytic Activities of Spinach Nitrate Reductase**

The effects of pH on the full and partial catalytic activities of nitrate reductase under conditions of constant ionic strength (µ = 50 mM) are shown in Figure 1. For the NADH-utilizing partial activities, NADH:FR, NADH:CR, and NADH:DR, both NADH:FR and NADH:DR activities exhibited pH optima of approximately 6.5, corresponding to rates of 224 and 132 µmol 2e/min/nmol heme, respectively, whereas the pH optimum for NADH:CR was shifted slightly higher, to approximately 7.3, corresponding to a maximum rate of 83 µmol 2e/min/nmol heme.

In contrast, the pH optima for the nitrate-utilizing activities, NADH:NR, FH$_2$:NR and MV:NR showed a somewhat wider range. The pH optimum for the physiological NADH:NR activity (Fig. 1B), determined at the optimal ionic strength of 50 mM, was found to be approximately 7, corresponding to a rate of 9 µmol 2e/min/nmol heme. Decreasing or increasing the pH to 5.5 or 9.5, resulted in retention of approximately 42% and 9% of the maximal activity, corresponding to rates of 3.74 and 0.81 µmol 2e/min/nmol heme, respectively.

At an ionic strength of 50 mM, FH$_2$:NR activity, using FADH$_2$ as the electron donor, exhibited a maximal rate of 12 µmol 2e/min/nmol heme with a pH optimum of approximately 7.5, activity decreasing to 1.08 and 5.52 µmol 2e/min/nmol heme at the pH extremes of 5.5 and 9.5, respectively. Under conditions of identical ionic strength, MV:NR activity

### Table II. Summary of Kinetic Constants of the Full and Partial Enzyme Activities of Spinach Nitrate Reductase

<table>
<thead>
<tr>
<th>Activity</th>
<th>$V_{max}$</th>
<th>$V_{max}$</th>
<th>$K_m$</th>
<th>$K_m$</th>
<th>$\mu$</th>
<th>pH $^*$</th>
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<tbody>
<tr>
<td>NADH:NR</td>
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<tr>
<td>NADH</td>
<td>9</td>
<td>5</td>
<td>7</td>
<td>6</td>
<td>50</td>
<td>7.0</td>
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<tr>
<td>NO$_3^-$</td>
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<td>ClO$_3^-$</td>
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<td>10751</td>
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<tr>
<td>NADH:FR</td>
<td>224</td>
<td>269</td>
<td>21</td>
<td>35</td>
<td>200</td>
<td>6.5</td>
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<tr>
<td>NADH:CR</td>
<td>83</td>
<td>76</td>
<td>6</td>
<td>29</td>
<td>50</td>
<td>7.3</td>
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<tr>
<td>NADH:DR</td>
<td>132</td>
<td>138</td>
<td>53</td>
<td>56</td>
<td>200</td>
<td>6.5</td>
</tr>
<tr>
<td>FH$_2$:NR</td>
<td>12</td>
<td>20</td>
<td>56</td>
<td>56</td>
<td>200</td>
<td>7.5</td>
</tr>
<tr>
<td>MV:NR</td>
<td>32</td>
<td>18</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>50</td>
<td>6.5</td>
</tr>
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</table>

$^*$ µ = 50 mM.  $^b$ µ = 200 mM.  $^c$ Values represent ionic strength or pH required for maximum activity.

![Figure 1](image-url)

**Figure 1.** Influence of pH on the full and partial activities of spinach nitrate reductase. A. Diaphorase activities, [NADH:FR (●), NADH:CR (□), and NADH:DR (△)]; B, nitrate-reducing activities [NADH:NR (○), FH$_2$:NR (△), and MV:NR (□)] were determined at 25°C under conditions of constant ionic strength (µ = 50 mM) at the indicated pHs as described in "Materials and Methods."
exhibited a maximal rate of 32 μmol 2e/min/nmol heme at pH 6.5 which decreased to 1.60 and 0.96 μmol 2e/min/nmol heme at pH 5.5 and 9.5.

**DISCUSSION**

The preceding results, summarized in Table II, provide the first detailed comparison of the maximal rates and Michaelis constants determined under optimum conditions for the full and partial enzyme activities catalyzed by spinach NR and the effects of changes in ionic strength and pH on these reactions. Previous reports that have attempted to characterize these activities for NR have utilized suboptimal conditions, such as the presence of effector anions (principally chloride and phosphate which have been shown to be inhibitors and nonessential activators of NR, respectively) or dithionite (including dithionite oxidation products) in the assay medium, suboptimal pH, use of impure enzyme or have failed to compare activities under equivalent conditions.

The results show that when the various activities of the spinach enzyme are compared under conditions of constant temperature (25°C), pH (7) and ionic strength (μ = 50 mm), and are normalized to reflect the transfer of 2 reducing equivalents for those activities using 1-electron acceptors (NADH:FR, NADH:CR) or 1-electron donors (MV:NR), they can be summarized by the following sequence:

NADH:FR > NADH:DR > NADH:CR

> MV:NR > FH₂:NR > NADH:NR

where NADH:FR and NADH:NR represent the fastest and slowest activities, respectively. Thus, for spinach NR, the full catalytic activity involving the maximum number of electron transfer steps (NADH to FAD to heme to Mo to NO₃⁻) represents the slowest rate. This sequence can be compared with the results of a similar study of the properties of the various activities of the *Chlorella* enzyme (10) which yielded a comparable sequence except that the rates for FH₂:NR and NADH:NR activities were equivalent, but still slower than any other partial activity. For *Chlorella* NR, this result has been interpreted in terms of the rate-limiting step in enzyme turnover involving electron transfer from the reduced heme to the Mo-pterin center. In contrast, the results for spinach NR indicate that none of the partial activities for this enzyme are as slow as the full activity which would indicate that the rate-limiting step in turnover of the spinach enzyme does not involve intramolecular electron transfer from reduced heme to Mo-pterin or electron transfer from reduced flavin to heme. However, activities that involved nitrate reduction were markedly slower than the diaphorase activities indicating the rate limitation may involve the Mo-pterin center in spinach NR.

The influence of ionic strength on the various activities indicates that they fall into two main groups, those that are enhanced by low ionic strength (NADH:NR, NADH:CR, and MV:NR) and those enhanced by high ionic strength (NADH:FR, NADH:DR, and FH₂:NR). Overall, increasing ionic strength was found to primarily affect activities involving the terminal Mo-pterin center. This grouping is slightly different for the influence of pH on the activities. Classical pH profiles were only observed for activities involving electron transfer between two or more prosthetic groups, with the exception of NADH:DR. The activities can be divided into two groups: NADH:FR, NADH:DR, and MV:NR activities exhibit pH optima of approximately 6.5 whereas the remaining three activities (NADH:NR, NADH:CR, and FH₂:NR) exhibit pH optima in the range 7 to 7.5. For the overall activity, the pH optimum is lower than previously reported (8) while increased ionic strength results in inhibition of activity. The nature of the zwitterionic buffers used in these assays, MES, MOPS, Tricine, or CHES, appeared to have little influence on any activity.

Comparison of the data obtained for the spinach and *Chlorella* NADH:NR activities showed contrasting effects of ionic strength. For the spinach enzyme, increased ionic strength resulted in decreased activity and an increased *Kₘ* for nitrate whereas *Chlorella* NR showed increased activity with increasing ionic strength (10).

Chlorate, bromate, and iodate could substitute for nitrate as the terminal electron acceptor. The halogenates, although exhibiting significantly higher *Kₘ*s, were found to be efficient acceptors when used under saturating conditions. Similar results have been shown for the *Chlorella* enzyme (13), although at longer time intervals significant product inhibition was observed.

**LITERATURE CITED**