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In Vitro Stability of Nitrate Reductase from Wheat Leaves

I. STABILITY OF HIGHLY PURIFIED ENZYME AND ITS COMPONENT ACTIVITIES[1]

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ABSTRACT

NADH-nitrate reductase has been highly purified from leaves of 8-day-old wheat (*Triticum aestivum* L. cv. Olympic) seedlings by affinity chromatography, using blue dextran-Sepharose 4B. Purification was assessed by polyacrylamide gel electrophoresis. The enzyme was isolated with a specific activity of 23 micromoles nitrite produced per minute per milligram protein at 25 C. At pH 7.5, the optimum pH for stability of NADH-nitrate reductase, this enzyme, and a component enzyme reduced flavin adenine mononucleotide ($FMNH_2$)-nitrate reductase has a similar stability at both 10 and 25 C. Two other component enzymes—methylviologen-nitrate reductase and NADH-ferricyanide reductase—also have a similar but higher stability. At this pH the Arrhenius plot for decay of NADH-nitrate reductase and methylviologen-nitrate reductase indicates a transition temperature at approximately 30 C above which the energy of activation for denaturation increases. $FMNH_2$-nitrate reductase and NADH-ferricyanide reductase do now show this transition. The energy of activation for denaturation (approximately 9 kcal per mole) of each enzyme is similar between 15 and 30 C. The optimum pH for stability of the component enzymes was: NADH-ferricyanide reductase, 6.6; $FMNH_2$-nitrate reductase and methylviologen-nitrate reductase, 8.9. All of our studies indicate that the NADH-ferricyanide reductase was the most stable component of the purified nitrate reductase (at pH 6.6, $t_{1/2}$ [25 C] = 704 minutes). Data are presented which suggest that methylviologen and $FMNH_2$ do not donate electrons to the same site of the nitrate reductase protein.

The enzyme nitrate reductase is a complex protein which not only catalyzes the reduction of nitrate to nitrite using NADH as an electron donor but is also capable of participating in two other enzymic reactions (1, 5, 6). The first of these is referred to as NADH-diaphorase and is measured by following the reduction of a suitable electron acceptor such as ferricyanide or Cyt *c*, using NADH as the reductant. The second component is capable of reducing nitrate to nitrite using either a reduced viologen dye, reduced flavin adenine dinucleotide, or $FMNH_2$[2] as the electron donor. These component enzymic activities are presumed to function sequentially in transferring electrons from NADH to nitrate (1, 5, 6).

The data presented in this paper arose from a general study of factors present in plant extracts which affect the *in vitro* stability and activity of NR. As an integral part of this study we routinely prepared highly purified NR by an affinity chromatography method described earlier for lactate dehydrogenase from rat liver by Ryan and Vestling (12) and later adapted for NR from *Chlorella vulgaris* (14) and *Glycine max* (2). It became apparent that enzyme prepared by this method differed in several important respects from NR prepared according to more conventional procedures (9). We wish to report on the comparative stability of the component enzymic activities and on the likely site of electron donation by $FMNH_2$ and MVH.

MATERIALS AND METHODS

PLANT MATERIAL

Sixteen seeds were sown in square plastic pots (8-cm sides) containing 300 g of a 1:1:2 mixture of Mt. Derrimut loam, course sand, and Perlite. The plants were irrigated every day with modified Hoagland solution containing 30 mM nitrate. The seedlings were grown in a controlled environment cabinet with a 12-hr 25 C day and a 12-hr 15 C night. Light was provided by Sylvania VHO cool-white fluorescent tubes and 40-w incandescent bulbs. The quantum flux (400–700 nm) at plant height was 190 $\mu E\ m^{-2}\ sec^{-1}$. The plants were harvested 8 days after sowing which corresponds with the time of maximum NR activity.

PURIFICATION OF NITRATE REDUCTASE

All purification steps were carried out at 4 C.

Preparation of Ammonium Sulfate Fraction. One-g primary leaves were cut into small pieces (approximately 4 mm in length) and ground with a pestle and mortar in 6 ml of extraction buffer containing 25 mM K-phosphate, 5 mM EDTA, and 5 mM L-cysteine HCl (pH 7.50). The homogenate was centrifuged at 25,000*g* for 15 min and solid ammonium sulfate (Ultra-pure; Schwarz/Mann) was slowly added to the supernatant with continuous stirring. The fraction precipitating between 30 and 45% saturation was dissolved in ice-cold extraction buffer so that the final volume was one-quarter of the volume of crude extract. When assayed immediately this fraction consistently yields at least 80% of the original total NR activity. In most experiments between 10 and 15 g of leaf tissue was used.

Blue Dextran-Sepharose 4B Affinity Chromatography. The ammonium sulfate fraction was loaded, without any further treatment, onto a column (2.3 × 8.0 cm) of blue dextran-Sepharose 4B equilibrated with extraction buffer. The column was then washed with 100 ml extraction buffer. NR was eluted from the gel by 5 μM NADH in extraction buffer. The flow rate during loading, washing, and elution was approximately 2 ml min^{-1}. The protein content of the crude extract and ammonium sulfate fraction was determined by the method of Lowry *et al.* (8) following precipitation with 12% (w/v) trichloroacetic acid. Protein in those fractions eluted from the blue dextran-Sepharose 4B was estimated according to the Lowry method, but only after they were each concentrated 20-fold by dialysis against PEG. Because of the

[1] This work was supported by the Wheat Industry Research Council of Australia and the Australian Research Grants Committee D2 74/15052.

[2] Abbreviations: E_a: energy of activation; FMN: flavin adenine mononucleotide; $FMNH_2$: reduced flavin adenine mononucleotide; MVH: reduced methylviologen; NR: nitrate reductase (EC 1.6.6.1).

extremely low concentration of protein (< 20 $\mu g\ ml^{-1}$) these measurements were at the limit of sensitivity of this assay method.

PREPARATION OF BLUE DEXTRAN-SEPHAROSE 4B

The procedure for preparing the activated gel is largely based on the methods described by Cuatrecasas *et al.* (3), Porath *et al.* (10), and Ryan and Vestling (12), and includes suggestions made by J. Goding, Walter and Eliza Hall Institute, Melbourne. Fifty ml of Sepharose 4B gel was washed on a sintered glass filter with 1,000 ml of water and suspended in 100 ml of 2 M sodium carbonate (pH 11.4). The suspension was stirred slowly with a glass rod and a solution of 10 g of cyanogen bromide dissolved in 10 ml of water-free dimethylformamide was added over 1 min. The suspension was slowly stirred with a glass rod for a further 2 min. The activated gel was immediately washed on a sintered glass filter with 500 ml of ice-cold 1 mM HCl. The activated gel was then immediately transferred to a solution of 1 g of blue dextran 2000 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) dissolved in 100 ml of 0.4 M sodium bicarbonate (pH 10.0), and the suspension gently mixed on a rotary tumbler for 18 hr at 4 C. The gel was washed on a sintered glass filter with 500 ml of distilled H_2O and suspended in 20 ml of 0.5 M Tris-HCl (pH 8.0) for 2 hr at 4 C to block unreacted activated sites. The gel was then washed successively with buffer of pH 4.0 (0.1 M citric acid-sodium hydroxide, 1 M potassium chloride) and pH 8.0 (0.1 M sodium citrate-citric acid, 1 M potassium chloride) until the A_{280} of the wash was negligible. Using this procedure approximately 13 mg blue dextran was coupled to each ml swollen gel.

ENZYME ASSAYS

NADH-NR. The reaction mixture contained 100 μmol K-phosphate (pH 7.50), 10 μmol potassium nitrate, 0.4 μmol NADH, and enzyme in a total volume of 2.0 ml. After 15 min incubation at 25 C, the reaction was stopped by adding 2 ml of a solution containing 0.5% (w/v) sulfanilamide and 0.1% (w/v) N-1-naphthylethylene diamine dihydrochloride in 1.5 N HCl; and after 15 min, nitrite was determined by measuring A at 540 nm.

MVH-NR. The reaction mixture contained 100 μmol potassium phosphate (pH 7.7), 10 μmol potassium nitrate, 1 μmol methyl-viologen, and enzyme in a total volume of 1.9 ml. The reaction was started by adding 0.1 ml of a freshly prepared solution of 10 mg/ml sodium dithionite dissolved in 95 mM sodium bicarbonate. After 15-min incubation at 25 C the reaction was terminated by vortexing which oxidized the MVH and any remaining dithionite. Nitrite was estimated according to the procedure described for NADH-NR.

$FMNH_2$-NR. The reaction mixture contained 50 μmol Tris-HCl (pH 8.5), 5 μmol potassium nitrate, 1.5 μmol FMN, and enzyme in a total volume of 0.9 ml. The reaction was started and terminated as described for the MVH-NR. Nitrite was estimated ac-

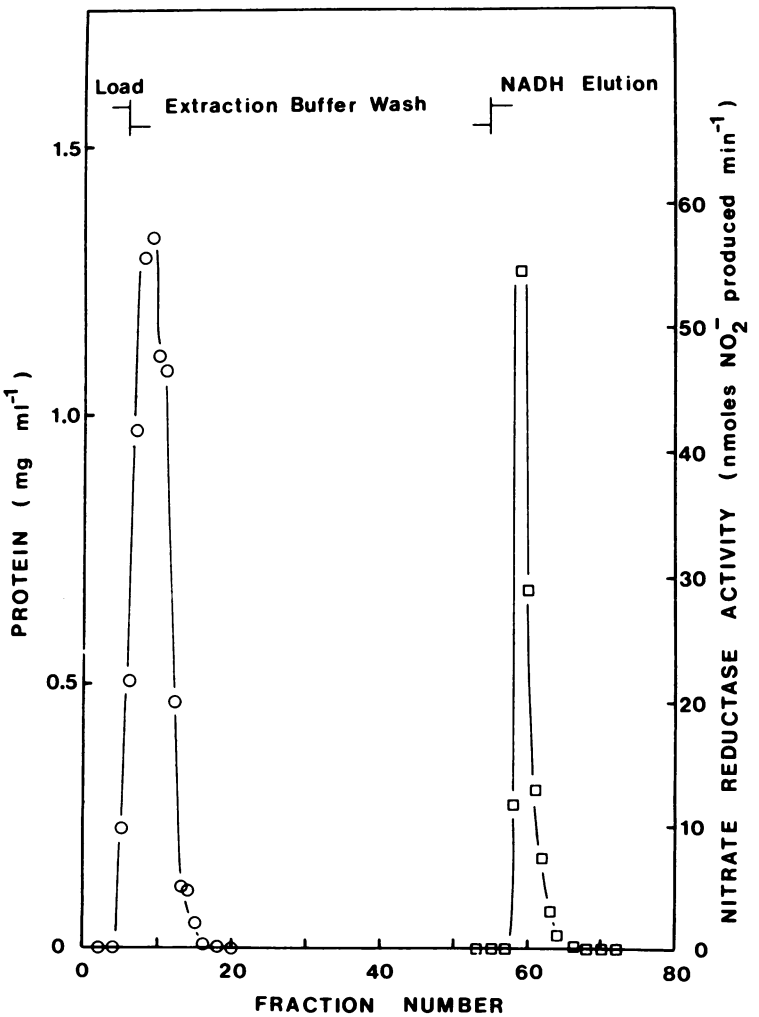

FIG. 1. Elution profile of NADH-NR from blue dextran-Sepharose 4B. Blue dextran column was loaded with 16 ml of the resuspended 30 to 45% ammonium sulfate fraction containing 31 mg protein. NR was eluted with 5 μM NADH in extraction buffer. Fractions of approximately 3 ml were collected from commencement of sample loading. Each fraction was then assayed to determine NR activity (□) and protein content (○).

cording to the procedure described for NADH-NR.

NADH-Ferricyanide Reductase. The reaction mixture contained 40 μmol Tris-HCl (pH 7.4), 0.7 μmol potassium ferricyanide, and enzyme in a total volume of 1.0 ml. The reaction was started by adding 0.05 ml NADH (4 mg/ml). Ferricyanide reduction was followed at 25 C by measuring the *A* change at 420 nm.

NADH-Cyt *c* Reductase. This assay was carried out only with enzyme prepared in the absence of cysteine. The reaction mixture contained 80 μmol potassium phosphate (pH 7.5), 1 mg Cyt *c* (Horseheart, Sigma), 0.3 μmol NADH, and enzyme in a total volume of 1.0 ml. The reaction was followed at 25 C by measuring the *A* change at 550 nm.

GEL ELECTROPHORESIS

Electrophoresis was carried out according to the method of Davis (4). Separation was carried out in 7.5% gels at 4 C. The pH of the electrode buffer was 8.3. The gels were stained with Coomassie blue.

CALCULATIONS

The E_a for denaturation was calculated according to the equation:

$$E_a = \frac{2.3\ RT_1T_2 \log \frac{k_2}{k_1}}{T_1 - T_2}$$

with k_1 and k_2 being the first order rate constants for NR decay at T_1 and T_2, where

$$k = \frac{0.693}{t_{1/2}}$$

The $t_{1/2}$ was calculated from the regression relating log residual activity and time of incubation.

RESULTS AND DISCUSSION

PURIFICATION OF NR

NADH-NR was eluted from blue dextran-Sepharose by concentrations of NADH as low as 1 μM, however 5 μM was used routinely (Fig. 1). Other compounds could also elute NR but they had to be used at a much higher concentration than NADH (Table I). Table II shows the results of a typical purification experiment. The over-all recovery was 21%, but this could be increased to approximately 40% if 10 μM FAD was included throughout the purification. The apparent recovery of NR was not improved by preincubation with ferricyanide prior to assay (data not shown).

The concentration of protein in the fractions eluted from the blue dextran-Sepharose was less than 1 μg/ml and hence was at the limit of sensitivity of the Lowry assay (see under "Materials and Methods"). The data shown for specific activity and degree of purification achieved are minimal estimates. Nevertheless the specific activity of 23.1 μmol nitrite produced min^{-1} mg $protein^{-1}$ at 25 C compares favorably with that reported for *G. max* of 0.4 μmol nitrate reduced min^{-1} mg $protein^{-1}$ at 30 C (2) and 86.2 μmol nitrate reduced min^{-1} mg $protein^{-1}$ at 20 C for *C. vulgaris* NR (14). Homogeneity of the NR prepared by this method was also difficult to assess because of the low amounts of protein

Table I. Effectiveness of different compounds in eluting NADH-nitrate reductase from Blue Dextran-Sepharose 4B.

Six identical columns (0.9 cm x 5.0 cm) containing Blue Dextran-Sepharose 4B were loaded with 5 ml of NR (30-45% ammonium sulphate fraction) containing 9 mg protein and washed with 100 ml of extraction buffer, NR was removed from the gel with each of the respective elutents.

Elutent	Concentration	Recovery[1]	Specific Activity[2]
		%	Units/mg protein
NADH	1 μM	23	24
NAD	Not eluted by 10 mM	—	—
NADPH	1 mM	13	8
NADP	Not eluted by 10 mM	—	—
KCl	200 mM	24	0.5
KNO_3	100 mM	19	2.6

1 Recovery is expressed as a per cent of total NR loaded.
2 One unit is 1 μmole nitrite produced per minute at 25C.

Table II. Purification of NADH-nitrate reductase from wheat leaves

Step	Total Activity	Protein	Specific Activity[1]	Purification	Recovery
	Units	mg	Units/mg	-Fold	%
Crude Extract	1.427	158.5	0.009	1.0	100
Ammonium Sulphate 30-45%	0.896	30.9	0.029	3.2	63
Blue Dextran	0.301	0.01	23.1	2573	21

1 One unit is 1 μmole nitrite produced per minute at 25C.

available. However only one band staining for Coomassie blue was found following disc gel electrophoresis (data not shown). This band was not tested for NR activity. Further support for the idea that NR prepared in this way is at a very high state of purification would include the very high specific activity observed and the extremely low level of protein eluted. In addition, the level of NADH required to elute NR from the blue dextran-Sepharose is very much lower (1 μM *cf.* 1 mM) than for other enzymes which are known to bind to the dye (16).

Some general properties of the purified enzyme are summarized in Table III.

FACTORS AFFECTING STABILITY OF PURIFIED NR

Effect of NADH, NO_3^-, and FAD. At 25 C the addition of 5, 10, or 20 mM NO_3^- or a combination of both NADH (200 μM) and NO_3^- had no effect on the stability of NADH-NR. With the addition of NADH alone, the stability of the enzyme was at first reduced but later became more stable than the control (Fig. 2). Inactivation of NR by NADH has been previously reported for spinach (9) and it appears that for wheat NR this effect is overcome by the addition of NO_3^-. FAD did not activate the purified enzyme but did increase its stability at 25 C from $t_{1/2}$ 30 min (minus FAD) to $t_{1/2}$ 70 min when incubated with FAD. The effect of FAD was independent of concentration within the range 5 to 100 μM. At 10 C FAD had no effect on stability.

Effect of pH. The effect of pH on the stability of NADH-NR is shown in Figure 3. The enzyme becomes increasingly unstable as the pH falls below 7.5. In addition, the kinetics of NR decay changes as the pH is lowered. At pH 7.5 NADH-NR appears to decay exponentially whereas at pH 7.0 or below decay is clearly nonexponential.

Whereas stability of the NADH-NR does not change greatly at pH greater than 7.0 (Figs. 3 and 4) the stability of the component enzymic activities: NADH-ferricyanide reductase, $FMNH_2$-NR, and MVH-NR, clearly do not show the same response to pH (Fig. 4). At 25 C NADH-ferricyanide reductase was found to possess maximum stability at pH 6.65 and minimum stability at pH 7.5 to 9.0. $FMNH_2$ and MVH-NR were found to have maximum stability at pH 8.9 with little change in stability over the range pH 6.3 to 7.5.

Effect of Temperature. At pH 7.5 decay of NADH-NR was exponential at all temperatures studied, with the $t_{1/2}$ varying from 84 min at 10 C to 1 min at 40 C. The Arrhenius plot for NADH-NR decay (Fig. 5) is complex with a definite inflection at approximately 30 C. Above this temperature the E_a for denaturation is considerably greater than the E_a calculated for the temperature range 10 to 30 C (75.3 kcal mol^{-1} *cf.* 9.9 kcal mol^{-1}). The majority of proteins normally have values ranging between 40 and 100 kcal mol^{-1} (17).

Arrhenius plots for decay of the other component enzymic activities of NR are shown in Figure 6. Data are presented for

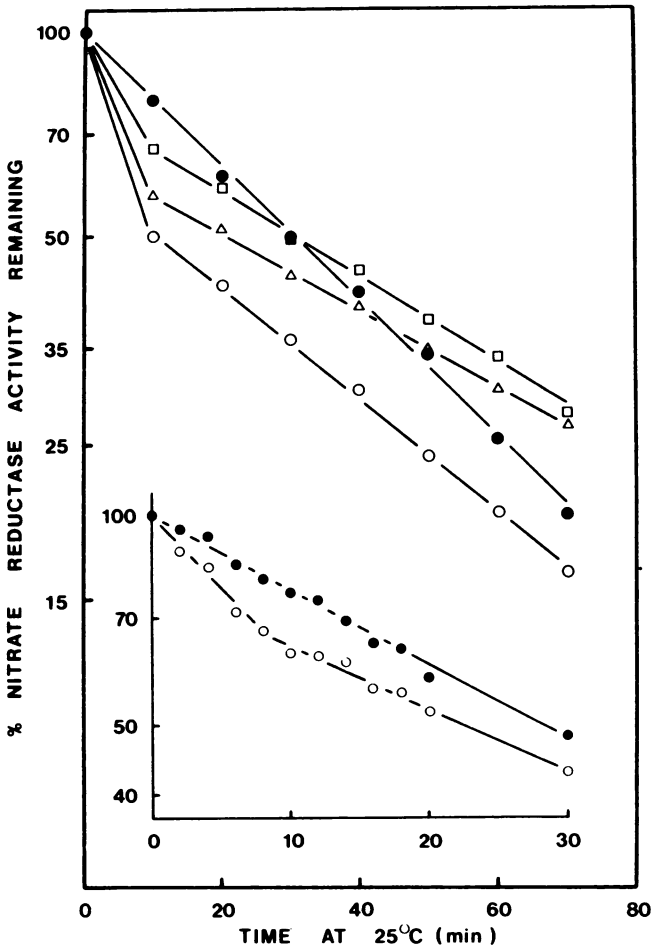

FIG. 2. Stability of NADH-NR in the presence of NADH. Purified NR (< 1 μg protein ml^{-1}) was incubated at 25 C in the presence of 0.2 mM (○), 0.4 mM (△) and 0.8 mM (□) NADH. Enzyme was also incubated in the absence of the cofactor (●). Stability was determined by incubating the purified enzyme at 25 C and withdrawing aliquots (100 μl) at regular intervals for determination of residual activity. Data are presented as a semilogarithmic plot.

Table III. Summary of some properties of purified wheat leaf nitrate reductase

The general conditions of the assay were the same as those described in "Methods". However, in the case of NADH-nitrate reductase the reaction was terminated by adding 100 μl of 50 mM zinc acetate and after 5 min the tubes centrifuged to remove the precipitate. Any NADH in the supernatant was oxidized by adding 100 μl of 261 μM phenazine methosulphate and incubating at 25C for 35 min (13). Nitrite was then estimated according to "Methods".

	Optimum Assay pH	*Km* Electron Donor	*Km* Electron Acceptor
NADH-nitrate reductase	7.5	3.3×10^{-5}M	3.4×10^{-4}M
MVH-nitrate reductase	7.7	8.8×10^{-7}M	1.3×10^{-3}M
$FMNH_2$-nitrate reductase	8.5	3.1×10^{-4}M	2.2×10^{-3}M
NADH-ferricyanide reductase	7.4	3.0×10^{-5}M	

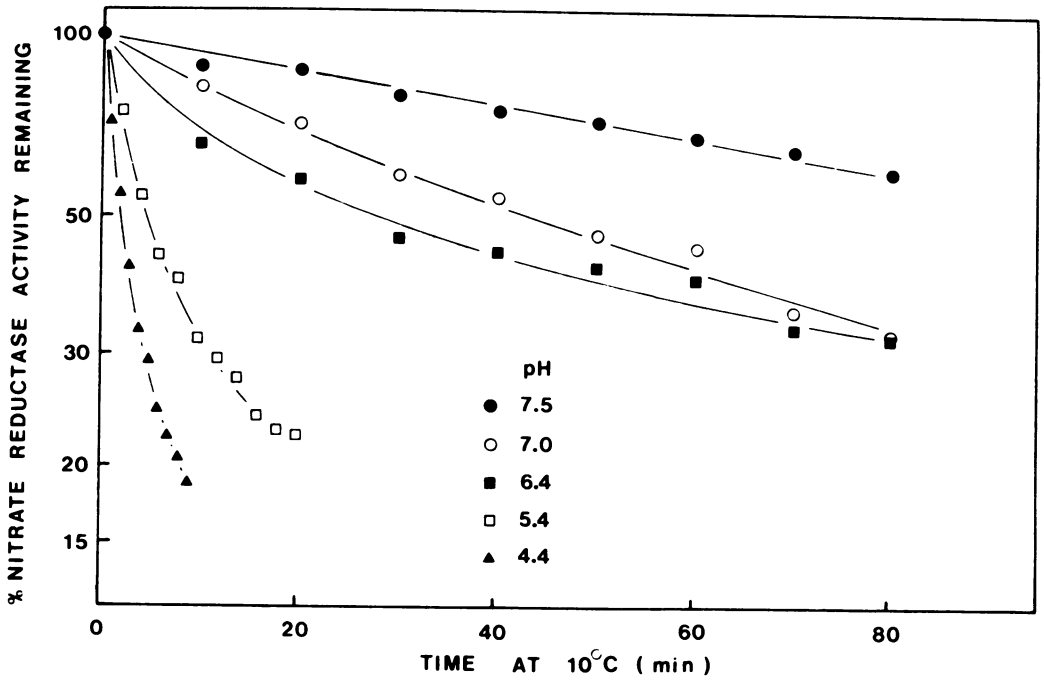

FIG. 3. Effect of pH on stability of NADH-nitrate reductase at 10 C. A range of 50 mM potassium phosphate buffers was prepared and equal volumes mixed with purified NR (< 1 μg protein ml^{-1}) to obtain final pH values of 4.4, 5.4, 6.4, 7.0, and 7.5. Each treatment was immediately assayed to determine initial activity, using a 200-μl aliquot. Treatments were then incubated at 10 C and aliquots withdrawn at regular intervals for determination of residual NR activity.

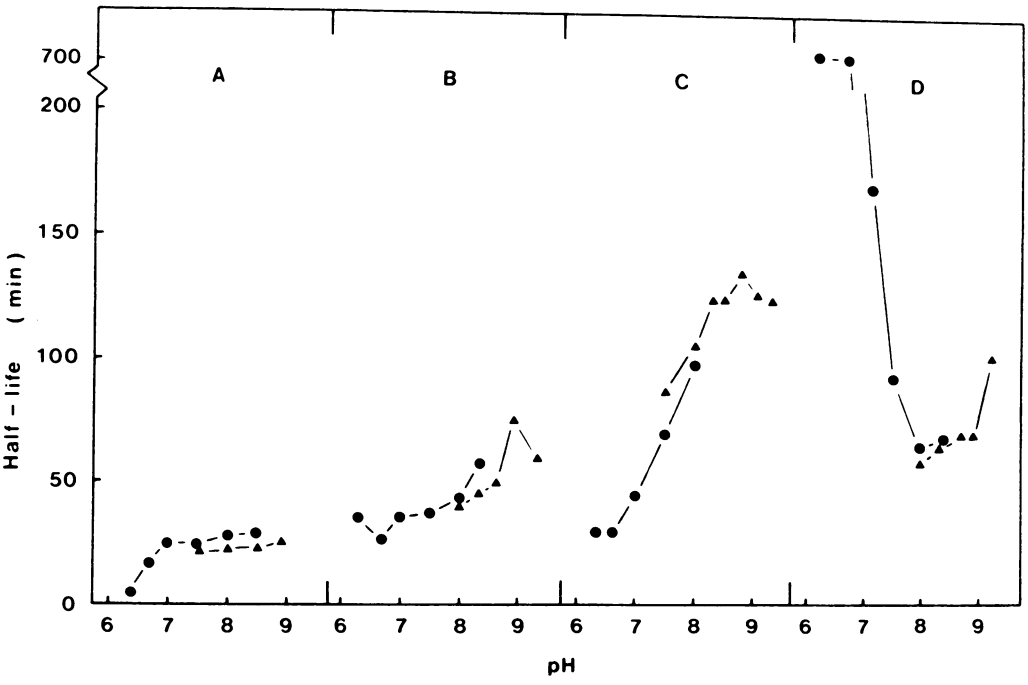

FIG. 4. Relation between pH and stability of NADH-NR (A), and the component enzymic activities, $FMNH_2$-NR (B), MVH-NR (C), and NADH ferricyanide reductase (D). Purified enzyme (< 1 μg protein ml^{-1}) was mixed with equal volumes of potassium phosphate buffer (●) or sodium borate buffer (▲) to obtain the treatment pH and assayed immediately using a 200-μl aliquot to determine initial activity. Treatments were then incubated at 25 C and aliquots withdrawn at regular intervals for determination of residual enzyme activity. The $t_{1/2}$ at each pH was determined according to the procedure outlined under "Materials and Methods."

decay measured at pH 7.5 and at the optimum pH for stability of each component.

pH 7.5. In the case of the NADH-ferricyanide reductase and $FMNH_2$-NR the Arrhenius plots are linear over the temperature range 10 to 42 C. The plot for the MVH-NR is discontinuous with a marked inflection at approximately 30 C. The E_a for denaturation of each enzyme is listed in Table IV.

Although the E_a for denaturation are very similar it should not be concluded that at pH 7.5 the NADH-NR and component enzymes have identical stabilities. The data show only that the relation between stability and temperature is virtually the same for the enzymes. At pH 7.5 stability of the NADH-NR and the

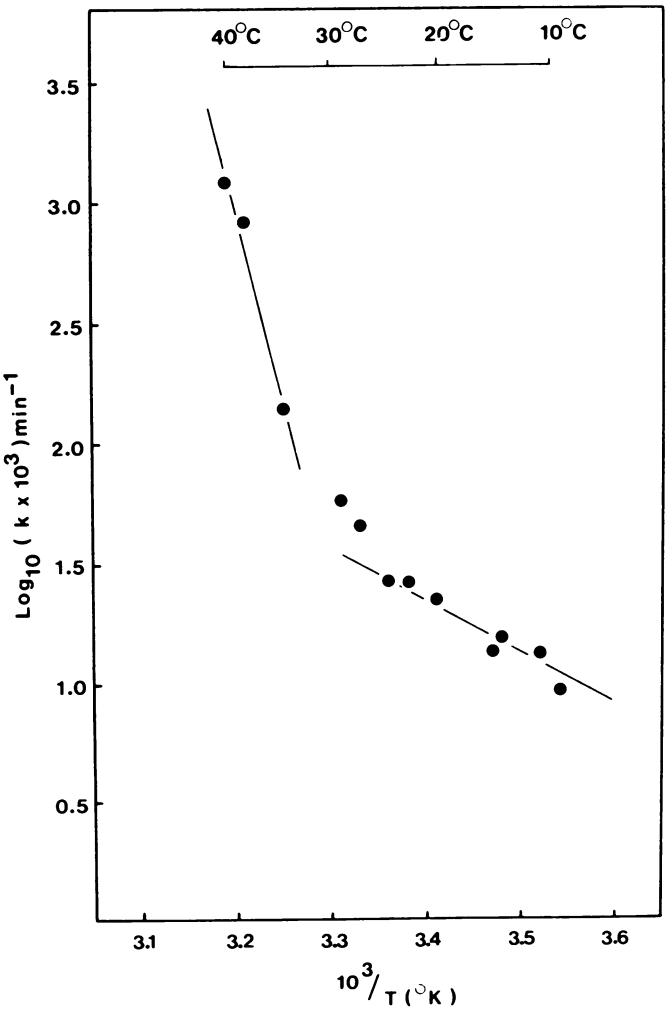

FIG. 5. Arrhenius plot for NADH-NR denaturation. Purified enzyme (< 1 μg protein ml^{-1}) was incubated at different temperatures within the range 10 to 42 C. At regular intervals 100-μl aliquots were withdrawn and the residual activity determined. The first order rate constant k was calculated from this data according to the procedure outlined under "Materials and Methods."

Table IV. E_a[1] for denaturation of NADH-nitrate reductase and component enzymic activities

E_a was calculated from data (Figs. 5 and 6) in the temperature range 10 to 25C, results are shown for pH 7.5 and the optimum pH for stability.

	pH 6.65	pH 7.50	pH 8.90
NADH-nitrate reductase	-	9.9	-
MVH-nitrate reductase	-	7.7	2.9
$FMNH_2$-nitrate reductase	-	9.7	2.5
NADH-ferricyanide reductase	27.6	9.5	-

1 kcal $mole^{-1}$

component activities fall into two groups of approximately equal stability (Table V), with the NADH-NR and $FMNH_2$-NR being the least stable.

Optimum pH. Stability was measured only in the temperature range 18 to 32 C. The E_a for denaturation of each enzyme is listed in Table IV.

Table V. Stability of NADH-nitrate reductase and component enzymic activities at pH 7.5.

Purified nitrate reductase (< 1 μg protein ml^{-1}) was incubated at 10 and 25C and at regular intervals the residual activity of NADH-NR and the component enzymic activities was determined using 100 μl aliquots. The pH was 7.5 in each case. The half-life was calculated according to the procedure described in "Methods".

	Half-life (min) 10C	25C
NADH-nitrate reductase	94	28
MVH-nitrate reductase	140	71
$FMNH_2$-nitrate reductase	90	33
NADH-ferricyanide reductase	140	93

The inflection observed in the Arrhenius plots for NADH-NR and MVH-NR is difficult to interpret, especially as the other components $FMNH_2$-NR and NADH-ferricyanide reductase did not show the same response. Inflections of this nature have previously been interpreted as being strongly suggestive of the enzyme having some association with a membrane (7, 11), the inflection reflecting a temperature-induced phase change in the membrane thereby affecting the enzyme. We have not examined this possibility nor can we offer any other explanation for our observations.

The strong temperature × pH interaction we observed when comparing stability of NADH-NR and the component enzymic activities has special significance to those studies in which stability differences have been used to develop ideas about the order of involvement of the different enzymic components in the transfer of electrons from NADH to nitrate. For example the most commonly accepted scheme for electron flow is shown in Figure 7 (adapted from refs. 1, 5, 6). According to this scheme electrons can be introduced and removed from the whole enzyme at several sites and this behavior is reflected in the component enzymic activities described in this report.

There are however several features of this concept of NR with which our results with the highly purified enzyme are in conflict. First, the site of acceptance of electrons from NADH is considered to be the most unstable (5, 6). Our results show that at pH 6.65 this portion of the enzyme is remarkably stable ($t_{1/2}$ 25 C = 704 min) and even at pH 7.5, where it has minimum stability, it is still very stable (Table V).

Inasmuch as this result was such a considerable departure from previously published reports (5, 6) we were wary of the possibility that a contaminating, but very stable, NADH-ferricyanide reductase had been coeluted from the blue dextran-Sepharose with NADH-NR. This does not seem to be the case as we have found that stability of NADH-ferricyanide reductase is very similar to that of NADH-Cyt *c* reductase, another proposed enzymic component of NR (data not shown). Additional evidence in support of the proposal that the NADH ferricyanide reductase activity is part of the NADH-NR protein includes the very similar K_m (NADH) of the two enzyme activities (Table III) and the competitive inhibition by ferricyanide of NADH-catalyzed nitrate reduction (data not shown). It is also unlikely that the high stability of the diaphorase component was due to the presence of cysteine in the elution buffer as the $t_{1/2}$ for both the NADH-NR and NADH-ferricyanide reductase are not affected when cysteine is omitted (data not shown).

With respect to the proposed site of acceptance of electrons from $FMNH_2$ and MVH, Hewitt *et al.* (6) suggested that this

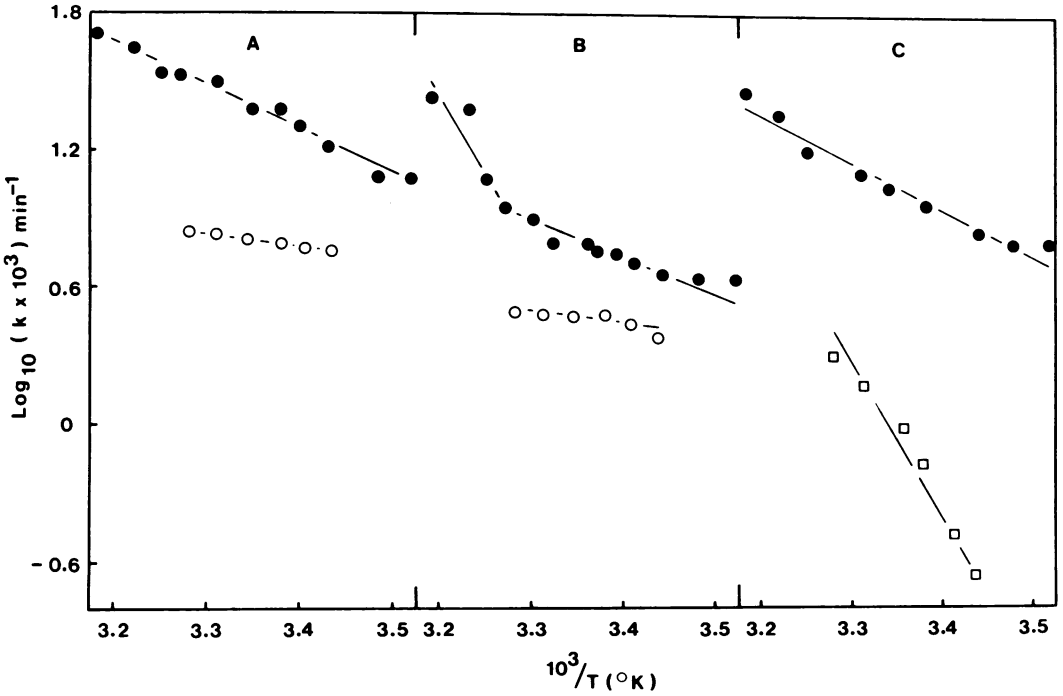

FIG. 6. Arrhenius plot for denaturation of $FMNH_2$-NR (A), MVH-NR (B), and NADH-ferricyanide reductase (C). Purified enzyme (< 1 μg protein ml^{-1}) was incubated at pH 7.5 (●) at different temperatures within the range 10 to 42 C or at pH 8.9 (○) and 6.65 (□) within the range 18 to 32 C. At regular intervals 100-μl aliquots were withdrawn and the residual activity determined for the indicated component enzyme. The first order rate constant k was calculated from these data according to the procedure outlined under "Materials and Methods."

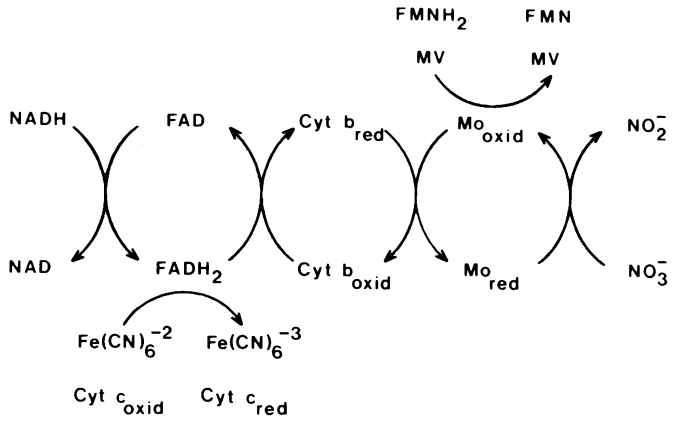

FIG. 7. Schematic diagram of electron flow through the NR enzyme complex.

occurs at Mo, with both MVH and $FMNH_2$ capable of reducing nitrate by way of electron transfer to Mo. If this were so then this component of the enzyme should be expected to show the same stability regardless of whether $FMNH_2$ or MVH is the source of electrons. This is not the case as the MVH system was considerably more stable (Table V, Fig. 4). Comparison of the Arrhenius plots for NR denaturation shows that MVH-NR denaturation exhibits a similar inflexion to that of NADH-NR (Figs. 5, 6) while the other components do not. These data also suggest that the MVH-NR and $FMNH_2$-NR components are different but no conclusion can be made as to the respective sites of electron donation by $FMNH_2$ or MVH. Whereas the data for comparative stability suggest that the MVH electron acceptance site is after that of $FMNH_2$ the Arrhenius plot data indicate the reverse.

We feel that these results can best be accommodated by a model for NADH-NR which incorporates two separate pathways of electron flow to nitrate, each distinct with respect to its optimum assay pH and affinity for the electron donor (Table III) and having different temperature sensitivities (Figs. 4–6, Table V). Electrons from NADH would presumably have equal access to either pathway. One of the pathways receives electrons from MVH and the other from $FMNH_2$. We cannot conclude whether the donors react with the same prosthetic group (FAD or Cyt *b*) or cofactor (Mo). Our results suggest that the site responsible for the instability of NADH-NR is likely to be between the site of exit of electrons to ferricyanide (probably from FAD) and the site of acceptance of electrons from either $FMNH_2$ or MVH.

Such a model would be consistent with the findings of Solomonson *et al.* (15) who reported that *Chlorella* NR contains 2 molecules each of FAD, Cyt *b*, and Mo and consists of three identical subunits each of approximately 100,000 mol wt. Although there are several possible arrangements of the subunits and components, the data nevertheless are strongly suggestive of the existence of parallel electron transfer pathways.

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