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Immunological Approach to Structural Comparisons of Assimilatory Nitrate Reductases[1]

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ABSTRACT

Homogeneous squash cotyledon reduced nicotinamide-adenine dinucleotide (NADH):nitrate reductase (NR) was isolated using blue-Sepharose and polyacrylamide gel electrophoresis. Gel slices containing NR were pulverized and injected into a previously unimmunized rabbit. This process was repeated weekly and antiserum to NR was obtained after four weeks. Analysis of the antiserum by Ouchterlony double diffusion using a blue-Sepharose preparation of NR resulted in a single precipitin band while immunoelectrophoresis revealed two minor contaminants. The antiserum was found to inhibit the NR reaction and the partial reactions to different degrees. When the NADH:NR and the reduced methyl viologen:NR activities were inhibited 90% by specifically diluted antiserum, the reduction of cytochrome *c* was inhibited 50%, and the reduction of ferricyanide was inhibited only 30%. Antiserum was also used to compare the cross reactivities of NR from squash cotyledons, spinach, corn, and soybean leaves, *Chlorella vulgaris*, and *Neurospora crassa*. These tests revealed a high degree of similarity between NADH:NR from the squash and spinach, while NADH:NR from corn and soybean and the NAD(P)H:NR from soybean were less closely related to the squash NADH:NR. The green algal (*C. vulgaris*) NADH:NR and the fungal (*N. crassa*) NADPH:NR were very low in cross reactivity and are apparently quite different from squash NADH:NR in antigenicity. Antiserum to *N. crassa* NADPH:NR failed to give a positive Ouchterlony result with higher plant or *C. vulgaris* NADH:NR, but this antiserum did inhibit the activity of squash NR. Thus, it can be concluded from these immunological comparisons that all seven forms of assimilatory NR studied here have antigenic determinants in common and are probably derived from a common ancestor. Although these assimilatory NR have similar catalytic characteristics, they appear to have diverged to a great degree in their structural features.

Assimilatory NR[3] is generally recognized as the catalyst for the initial step of nitrate assimilation where nitrate is reduced to nitrite with pyridine nucleotide as electron donor (10). Assimilatory NR are accepted to be of large molecular size, and to contain heme-iron, flavin-adenine dinucleotide, and molybdenum. These components act as electron carriers between the NADH oxidation site and the nitrate reduction site (5). NR is also capable of acting as an NADH dehydrogenase, catalyzing the reduction of ferricyanide, mammalian Cyt *c*, and other acceptors, and can also catalyze the reduction of nitrate with reduced methyl and benzyl viologen and reduced flavins as electron donors (22).

Although all assimilatory NR are capable of catalyzing the same types of reactions, current data indicate that the specific structure of NR is species dependent. NADH:NR from *Chlorella vulgaris* has been reported to have a mol wt of 300 KD with 3 subunits, each with a mol wt of 90 to 100 KD (7, 23). NADPH:NR from *Neurospora crassa* has a mol wt of 230 KD with 2 subunits, each with a mol wt of 115 KD (19). The structures of higher plant NR have been less fully characterized. NADH:NR from spinach has been reported to have a mol wt of 197 KD, with a multitude of subunit sizes ranging from 37 to 160 KD (16). NADH:NR from squash cotyledons has a mol wt of 200 KD, while having 35 KD mol wt subunits (4). Even discrepancies concerning the composition of NR from the same species exist, as one group has reported that NADH:NR from barley leaves showed a subunit mol wt of 100 KD (13), while others reported the presence of 40 and 60 KD subunits (21). Although the differences in the methods used to study the mol wt of these NR may explain some of this disparity of results, it appears that assimilatory NR from different species may be structurally different. The differences observed in structural features are also apparent in other properties of NR. The "typical" higher plant NR is specific for NADH and has a pH optimum of 7.5 (10). However, soybean leaves possess two NR which are different from other higher plant NR (3). Both NR are capable of catalyzing nitrate reduction with either NADH or NADPH, and show a pH optimum of 6.5 (3). The NAD(P)H:NR is bispecific for pyridine nucleotides being more active with NADPH than with NADH, and has a high K_m for nitrate (3). It has been found, however, to have the same two-site ping pong kinetic mechanism as previously reported for NR from squash cotyledons and corn leaves (5, 17). The soybean NADH:NR has a K_m for nitrate typical of most higher plant NR, and a six to one preference for NADH *versus* NADPH (3). Assimilatory NR appear to have significantly different molecular structures while retaining the same prosthetic groups and many of the same catalytic characteristics. Higher plant, algal, and fungal NR appear to represent a homologous series of eukaryotic enzymes that have diverged from a common ancestor.

Another means to study the chemical similarity and structural homology of proteins is through the use of immunological cross reactions. These studies have been used to examine evolutionary divergence of proteins such as lysozyme, catalase, trypsin, and Cyt *c* (2). In addition, structural comparisons have been performed using antiserum to ribulose-1,5-bisphosphate carboxylase (9, 14). It has recently been reported that the majority of evolutionary substitutions in proteins are immunologically detectable (24). Therefore, it seems useful to compare immunologically the assimilatory NR from several species. Monospecific antisera have been prepared against NR from *C. vulgaris* and *N. crassa* (1, 6). Impure

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[3] Abbreviations: NR, nitrate reductase; KD, kilodaltons; anti-NR (source), antibodies directed against nitrate reductase from the source indicated.

antisera have previously been prepared against NR from spinach and *Aspergillus nidulans* (8, 15). We describe here the preparation of monospecific antiserum against squash cotyledon NADH:NR, and detail the use of the antiserum to compare immunologically the similarity between squash cotyledon NR and other assimilatory NR obtained from spinach, corn, soybean, *N. crassa*, and *C. vulgaris*.

MATERIALS AND METHODS

Growth of Plants and Alga. Squash (*Cucurbita maxima* L. cv. Buttercup) and corn (*Zea mays* L. cv. W64A × W182E) were grown as previously described (5) and induced with 100 mM KNO_3 and 1 mM $(NH_4)_2SO_4$ in Hoagland solution 12 h before harvest. Spinach (*Spinacia oleracea* L.) were grown in the greenhouse in vermiculite for 6 weeks, subirrigated every other day with Hoagland solution, and induced as described above. Soybean (*Glycine max* L. Merr. cv. Prize) were grown and induced as previously described (3). *C. vulgaris* cells were grown for 3 days under continuous illumination as previously described with nitrate as the sole nitrogen source (23). The original culture was a gift from Dr. L. P. Solomonson, University of South Florida.

Preparation and Assay of NR. NADH:NR (E.C. 1.6.6.1) was purified from squash cotyledons and corn leaves using blue-Sepharose as previously described (5). Blue-Sepharose purified squash cotyledon NADH:NR was further purified to electrophoretic homogeneity using Procion Red-Sepharose, as previously described (4). NADH:NR was purified from spinach leaves using blue-Sepharose as described for corn leaf NR (5). The NAD(P)H:NR (E.C. 1.6.6.2) and the NADH:NR from soybean leaves were prepared using blue dextran-Sepharose according to Campbell (3). NADH:NR from *C. vulgaris* was purified using blue-Sepharose as described by Funkhouser and Ramadoss (6). NADPH:NR from *N. crassa* (E.C. 1.6.6.3) was a gift of Dr. R. H. Garrett, University of Virginia. All NADH:NR were assayed as previously described (3, 22), while *N. crassa* NR was assayed by substituting 0.1 mM NADPH for NADH. The soybean leaf NAD(P)H:NR was assayed at pH 6.5 with 80 mM nitrate and 0.1 mM NADPH (3). Reduced methyl viologen:NR, NADH:Cyt *c* reductase, and NADH:ferricyanide reductase activities of squash cotyledon NR were assayed as previously described (22). One unit of activity equals 1 μmol substrate converted/min. Protein was measured as previously described using BSA (Fraction V, Sigma) as the standard (22).

Production of Antisera. Squash cotyledon NADH:NR was isolated using blue-Sepharose and polyacrylamide gel electrophoresis as previously described (22). NR activity was localized on the gels (23), and gel sections were excised, pooled, and pulverized in a glass homogenizer with approximately 5 ml of 10 mM K-phosphate (pH 7.5), and 0.15 M NaCl. This suspension was subsequently emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously into a previously unimmunized rabbit. The NR was prepared weekly and injected into the rabbit in the same manner except the mixture was emulsified with Freund's incomplete adjuvant. Approximately 100 μg of homogeneous NR, as determined by protein assays, was injected into the rabbit each week. After 4 weeks, and at regular intervals thereafter, whole blood was obtained by cardiac puncture and serum was collected after centrifugation. The antiserum was used without further purification. Serum from an unimmunized rabbit was used as the control. Anti-NR (*N. crassa*) was a gift of Dr. R. H. Garrett, University of Virginia.

Gel Immunodiffusion and Immunoelectrophoresis. Immunodiffusions were performed in two dimensions as described by Ouchterlony and Nilsson (18). Noble agar (1%) (Difco) slabs were prepared in 10 mM K-phosphate (pH 7.5), and 0.8% NaCl. Precipitin bands were allowed to develop 36 h at 22 C in a humid environment, and unreacted protein was removed from the gels with 1 day wash with 0.15 M NaCl, followed by a thorough rinse with deionized H_2O. Precipitates were stained for 0.5 h with 1% Coomassie Brilliant Blue in acetic acid:ethanol:water (10:45:45), and destained with acetic acid:ethanol:water (10:45:45).

Immunoelectrophoresis was carried out in 2% agar in 50 mM barbital buffer (pH 8.6). Barbital buffer (50 mM) also comprised the reservoir buffer. Electrophoresis was run at 80 v for approximately 2 h. Antisera were subsequently added to the center trough, and precipitin bands were allowed to develop 24 h at 22 C. The slides were washed and stained as described above.

RESULTS

Characterization of the Antiserum. The precipitation reaction between anti-NR (squash) and blue-Sepharose purified squash cotyledon NADH:NR was examined using the Ouchterlony gel diffusion system (Fig. 1). Figure 1A shows the reaction of increasing concentrations of NR (outer wells) with a constant volume of antiserum (center well), whereas Figure 1B shows the reaction of increasing concentrations of antiserum (outer wells) with a constant quantity of NR (center well). In each case a single precipitin band was observed with a line of identity between the linearly diluted samples. Squash cotyledon NADH:NR, purified to electrophoretic homogeneity using Procion Red-Sepharose (4), formed a single precipitin band with the antiserum and showed a line of identity with blue-Sepharose purified NR using the double diffusion system. Control serum gave no precipitin reaction with blue-Sepharose purified NR.

Immunoelectrophoresis was performed on blue-Sepharose purified and Procion Red-Sepharose purified squash cotyledon NR (data not shown). A single major protein staining band was observed for both preparations of purified NR, while two minor contaminants were found to cross-react in the blue-Sepharose fraction. The bands for the contaminants were too faint to establish clearly the lines of identity with the major NR band but aggregation of NR or other artifacts of electrophoresis could account for these minor bands (4).

Anti-NR (*C. vulgaris*) and anti-NR (*N. crassa*) have been found to inhibit their respective NR activities (1, 6). Anti-NR (squash) was diluted linearly, incubated with a constant amount of blue-Sepharose purified NR, and examined for the effect on the NADH:NR, NADH:Cyt *c* reductase, NADH:ferricyanide reductase, and reduced methyl viologen:NR activities (Fig. 2). These activities were found to be differentially inhibited by antiserum. The NADH:NR and the reduced methyl viologen:NR were most susceptible to inhibition by antiserum, as only 10% of these activities remained after incubation with antiserum diluted approximately 20-fold. At this same degree of dilution of the antiserum, 50% of the NADH:Cyt *c* reductase activity remained after incubation, whereas 70% of the NADH:ferricyanide reductase activity was still present. Control serum at 10 times the maximum concentration used in this study had no effect on the NADH:NR activity.

Immunological Comparisons of Assimilatory Nitrate Reductases. Ouchterlony double diffusion gels were used to analyze the cross reactivity of anti-NR (squash) with NR isolated and purified from several sources. The results of these analyses are schematically represented in Figure 3. For NR from spinach and corn, a single precipitin band was found for each which was partially fused to the band for squash NR. Although these higher plant NR share some antigenic determinants with squash NR, they are not identical in structure. NR from *C. vulgaris* showed no precipitin reaction with anti-NR (squash). A previous report indicated that anti-NR (*C. vulgaris*) gave a negative Ouchterlony result with spinach NR (6). This algal NR appears to share few antigenic determinants with higher plant NR. In other Ouchterlony assays, we found no precipitin reaction between anti-NR (squash) and NR from *N. crassa*. In addition, anti-NR (*N. crassa*) did not give

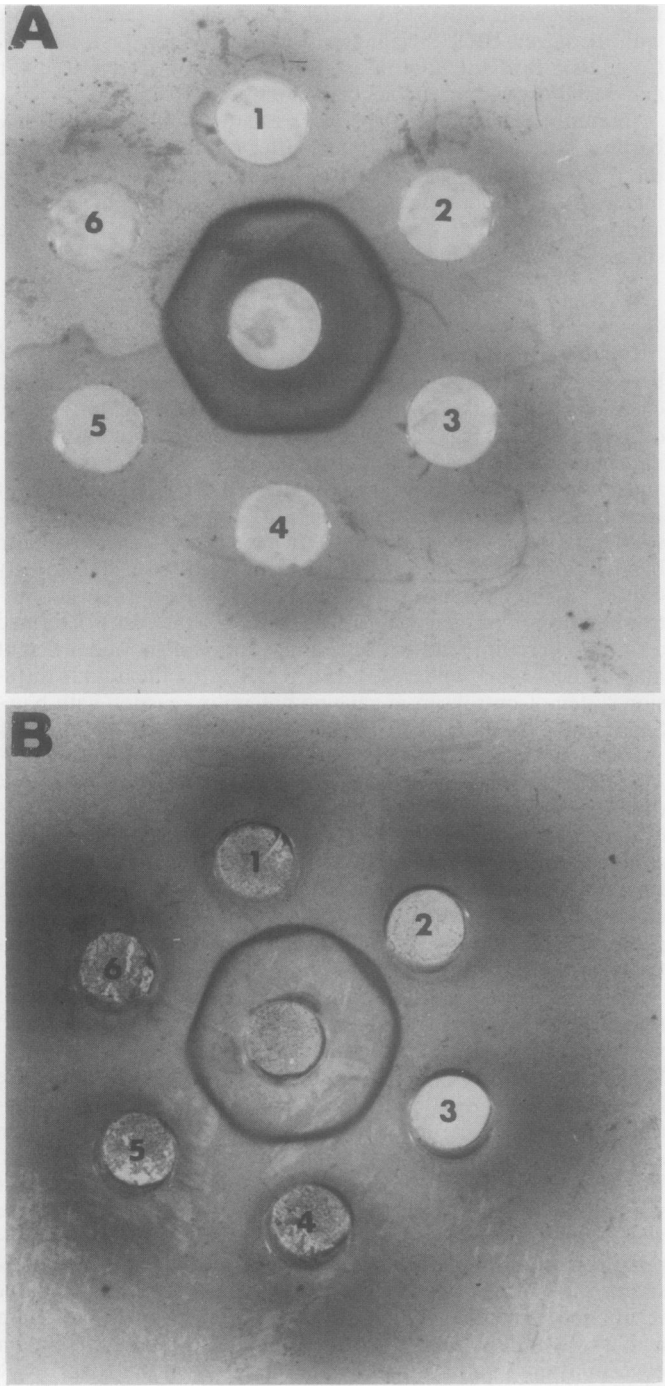

FIG. 1. Demonstration of monospecific antiserum to squash cotyledon NADH:NR with Ouchterlony double diffusion. Twenty microliter wells were cut in the agar. Each center well was filled once. The surrounding wells were filled the number of times indicated. 1A, antiserum in center well, NR in surrounding wells; 1B, NR in center well, antiserum in surrounding wells. The activity of the NR sample was 5.7 units/ml.

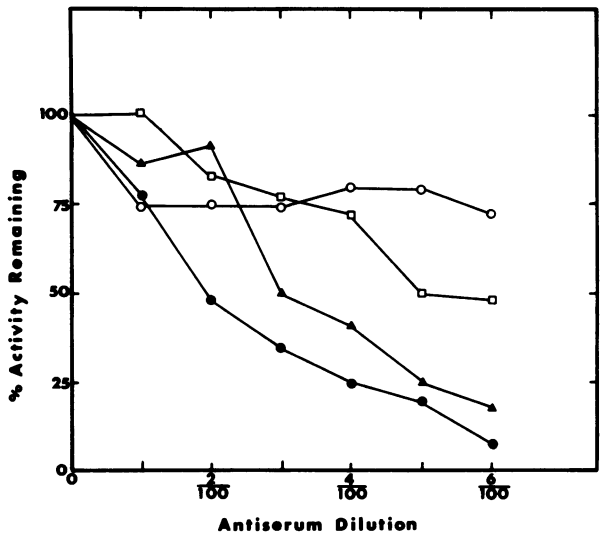

FIG. 2. Inhibition of squash NADH:NR and associated activities by anti-NR (squash). A 0.3-ml sample of blue-Sepharose purified NR (1.7 units/mg protein) was incubated with 0.1 ml of antiserum diluted in 40 mM K-phosphate, (pH 7.4) and 0.15 M NaCl as shown. After 10 min at 20 C, aliquots of these mixtures were assayed for the various activities. One hundred percent equaled 0.07 units of NADH:NR activity. NADH:NR (●——●); NADH:Cyt *c* reductase (□——□); NADH:ferricyanide reductase (○——○); and reduced methyl viologen:NR (▲——▲).

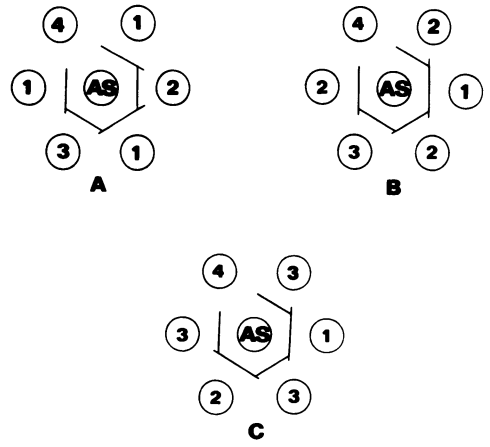

FIG. 3. Schematic representation of comparative ouchterlony double diffusion analyses of nitrate reductases purified from several sources. Twenty μl wells were cut in the agar. Wells were filled with NR samples as shown: well 1-squash (4.4 units/ml); well 2-spinach (2.9 units/ml); well 3-corn (0.6 units/ml); and well 4-*C. vulgaris* (3.2 units/ml). AS indicates anti-NR (squash) in the center well.

a positive Ouchterlony assay result with NR from squash, spinach, corn, or *C. vulgaris*. Anti-NR (*C. vulgaris*) was also reported not to give a positive result with NR from *N. crassa* in the Ouchterlony assay (6). NR from *N. crassa* appears to share few antigenic determinants with these four NR's.

The results of the Ouchterlony double diffusion assays were used to examine antigenic similarities among the higher plant NR. Figure 3A shows the partial fusion of precipitin bands occurring between the squash and spinach NR and between the squash and corn NR. A spur occurs in the direction of the spinach NR in the first case and the corn NR in the second. When comparing the precipitin reaction of two antigens with a single antiserum, a spur will be found to extend toward the antigen which is less closely related to the antiserum (18). Thus, NR from spinach and corn contain fewer cross-reacting determinants than squash NR. Figure 3B depicts the comparison of the spinach NR with the other NR. Partial fusion occurs between the spinach-squash and the spinach-corn NR. The spur formation is in the direction of the spinach NR for the first band and in the direction of the corn NR for the second precipitin band. Thus, it appears that the spinach NR is more closely related to the squash NR than the NR from corn. The results in Figure 3A and B are further confirmed by Figure

3C, in which partial fusion of precipitin bands was found to occur between the corn-squash and corn-spinach NR, with the spur formation directed toward the corn NR in both cases.

Inasmuch as anti-NR (squash) was found to inhibit significantly squash NADH:NR activity, we examined the effect of linearly diluted antiserum on the activity of different quantities of NR (Fig. 4). A family of curves was generated in which the highest concentration of NR was least affected by the linearly diluted antiserum. This type of analysis is similar to the precipitation curves obtained when antigens are reacted with antibodies, and the amount of antibody-antigen complex is measured (11). In the case of NR we equate the amount of bound antigen with inhibition of enzyme activity. These analyses can be used to screen for the cross-reactivity of antigens with a single antiserum (11).

Linearly diluted antiserum was used to inhibit the NADH- and NADPH-linked activity of NR from different sources (Fig. 5). The inhibition of similar amounts of NR activity units from squash, spinach and corn indicated that squash and spinach NADH:NR were similar in antigenicity, while corn NADH:NR was less similar (Fig. 5A). Although the amount of *C. vulgaris* NADH:NR used was about one-third the squash NR, only about 35% inhibition of the algal enzyme was found at the highest concentration of antiserum. If these proteins shared many antigenic determinants, *C. vulgaris* NR would have been more strongly inhibited by the linearly diluted antiserum than was observed. However, increasing the concentration of anti-NR (squash) approximately ten-fold led to complete inhibition of *C. vulgaris* NR activity (data not shown). Since anti-NR (squash) inhibited *C. vulgaris* NR, the algal NR has some antigenic determinants in common with squash NR. This result is in agreement with the previously reported inhibition of spinach NR by anti-NR (*C. vulgaris*) (6). The NADH and NAD(P)H:NR from soybean primary leaf were analyzed at activity levels somewhat less than the squash NADH:NR activity (Fig. 5B). If the soybean NR were similar in antigenicity to squash NR, a greater degree of inhibition would have been expected than was observed. Because of the different amounts of activity of soybean NR available for these assays, it cannot be concluded whether one of the soybean enzymes is more closely related to squash than the other. Finally, the inhibition of *N. crassa* NADPH:NR was analyzed at an activity level of one-half the level of squash NADH:NR activity (Fig. 5B). Here again, a greater degree of inhibition of the *N. crassa* NR would have been expected if the two enzymes shared many antigenic determinants. However, *N. crassa* and squash NR appear to have some determinants in common. This was confirmed by showing that anti-NR (*N. crassa*) inhibited the squash NADH:NR activity (data not shown). It was previously reported that anti-NR (*C. vulgaris*) inhibited the activity of *N. crassa* NR (6).

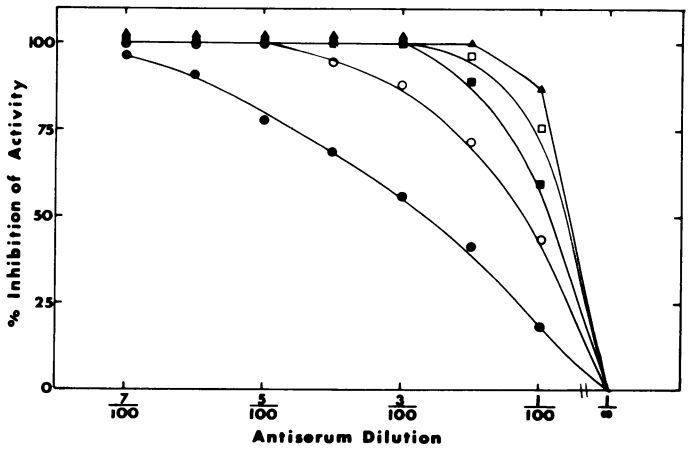

FIG. 4. Anti-NR (squash) inhibition of squash NADH:NR activity. A 0.3-ml sample of blue-Sepharose purified NR (1.4 units/mg protein) was incubated for 10 min with 0.1 ml linearly diluted anti-NR (squash). Immediately after incubation at 22 C, aliquots were assayed for activity. Activities of diluted NR samples were: 0.008 units (▲——▲); 0.013 units (□——□); 0.018 units (■——■); 0.029 units (○——○); and 0.060 units (●——●).

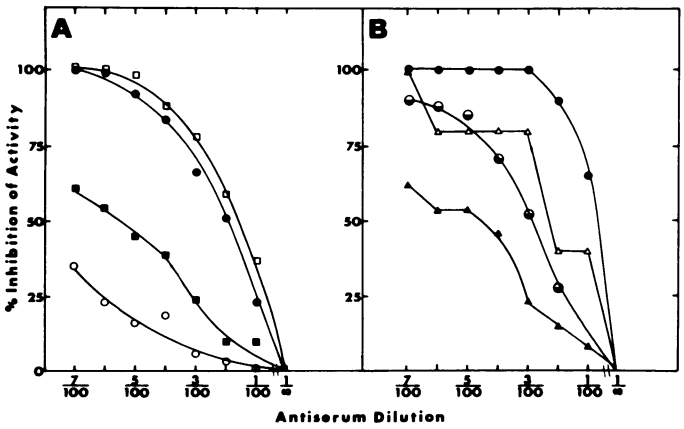

FIG. 5. Anti-NR (squash) inhibition of nitrate reductase activity purified from several sources. Incubations of enzyme samples with diluted anti-NR (squash) were done as described in Figure 4 and assays for activity were done as described in the text. Activities of samples for A were: Squash NADH:NR-0.052 units (●——●); Spinach NADH:NR-0.026 units (□——□); Corn NADH:NR-0.036 units (■——■); and *C. vulgaris* NADH:NR-0.016 units (○——○). Activities of samples for B were: Squash NADH:NR-0.01 units (●——●); *N. crassa* NADPH:NR-0.005 units (◒——◒); Soybean NADH:NR-0.003 units (△——△); and Soybean NAD(P)H:NR-0.007 units (▲——▲).

DISCUSSION

We have found that μg quantities of electrophoretically homogeneous squash cotyledon NADH:NR were sufficient to elicit an antibody response in an unimmunized rabbit. By Ouchterlony double diffusion analyses, the antiserum against squash cotyledon NR was apparently monospecific. However, immunoelectrophoresis revealed the presence of two minor contaminants which may be the result of the anomalous behavior of NR during electrophoresis. NR has been found to show aberrations in size when exposed to different electrophoretic conditions (4). Monospecific antisera have previously been prepared against NR from *N. crassa* and *C. vulgaris* (1, 6), while partially purified NR from spinach has been found to elicit an antibody response in guinea pigs (8, 15). Crude NR from *A. nidulans* has also been found to elicit antibodies in rabbits (20).

The anti-NR (squash) inhibited all the activities of the squash NADH:NR. However, the activities involving reduction of nitrate were inhibited to a greater degree than those which involved only the dehydrogenase function of the enzyme. These results indicate that the antiserum contained antibodies to different antigenic sites of the squash NR. The NADH:Cyt *c* reductase activity of NR was inhibited to a greater degree than the NADH:ferricyanide reductase activity. This difference in degree of inhibition of NADH dehydrogenase activities may only be apparent, since the smaller ferricyanide may have had greater access to the reduced enzyme-antibody complex than the larger Cyt *c* (1). For sulfite oxidase, a differential inhibition of activity with artificial electron acceptors was also reported (12). For this oxidase, the sulfite-linked reduction of ferricyanide was not inhibited by antibody whereas the reduction of Cyt *c* was inhibited (12). The present results with anti-NR (squash) more closely resemble those obtained by Amy and Garrett (1) with anti-NR (*N. crassa*) than those of Funkhouser and Ramadoss (6) with anti-NR (*C. vulgaris*). The anti-NR (*C.*

vulgaris) was reported to inhibit totally the NADH:NR, NADH: Cyt *c* reductase, and reduced methyl viologen:NR activities of the algal enzyme (6).

Antiserum to squash cotyledon NADH:NR was used to compare immunologically the cross reactivities of pyridine nucleotide-linked NR's. Ouchterlony double diffusion analyses and inhibition of activity were used as a basis for this comparison. As expected, squash cotyledon NR was found to be the most antigenic as revealed by Ouchterlony assay and inhibition of activity. However, inhibition of activity assays indicated that all six forms of assimilatory NR studied here have antigenic sites in common with squash NR. Spinach NADH:NR appeared to be the most closely related to squash NR, as revealed by both the Ouchterlony and inhibition of activity assays. The other higher plant NR studied were less closely related to squash NR, but it was not possible to establish an order of relatedness from the current results. *C. vulgaris* NADH:NR appeared to be less closely related to the squash NR than any of the higher plant NR which was most clearly shown by the Ouchterlony assays. *N. crassa* NADPH:NR was also shown to be not related closely to the higher plant NR by analyses with both the anti-NR (squash) and the anti-NR (*N. crassa*). Previous studies with the anti-NR (*C. vulgaris*) indicated that *C. vulgaris* NR had common antigenic determinants with spinach and *N. crassa* NR but that these enzymes were not closely related (6).

The extent of cross-reaction between enzymes and antibodies appears to depend on the phylogenetic distance between the organisms from which the enzymes were isolated (2). In addition, it has been suggested that when a substitution occurs in an amino acid residue of a protein, the antigenicity of the protein is affected (24). The degree of cross reactivity of the NR forms appears to correlate roughly with the phylogenetic distances among higher plants, algae and fungi. Thus, these NR may represent a homologous series of divergent proteins, maintaining similarity in catalytic properties while drifting apart at nonessential residues. Alternatively, during the evolution of NR to the modern forms of the enzyme, specific alterations of the enzyme's structure may have occurred which were related to the physiology and biochemistry of the organisms. It seems likely that both forces may have influenced NR antigenicity. The general descriptive properties of NR activity, including catalytic activities and kinetic mechanism, indicate that the pyridine nucleotide NR are divergent forms of a common ancestral NR. However, structural differences between NR forms do not conform with this view since higher plant NR has subunits with a mol wt of 35 to 40 KD (4, 16, 21), while algal and fungal NR have subunits with mol wt of 90 to 115 KD (7, 19). These large differences in structural characteristics of the NR family indicate that these proteins may not share a common origin. In contrast, the immunological studies described here indicate that higher plant, algal, and fungal NR are structurally related and share a common origin. To resolve the question of the origin of the NR polypeptides, detailed knowledge of the chemical structures of these polypeptides, or of the nucleic acid sequences coding for them, will be required.

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LITERATURE CITED

1. Amy NK, RH Garrett 1979 Immunoelectrophoretic determination of nitrate reductase in *Neurospora crassa*. Anal Biochem 95: 97–107
2. Arnon R 1973 Immunochemistry of enzymes. *In* M Sela, ed, The Antigens, Vol I. Academic Press, New York, pp 97–159
3. Campbell WH 1976 Separation of soybean leaf nitrate reductases by affinity chromatography. Plant Sci Lett 7: 239–246
4. Campbell WH, MG Redinbaugh, WB Mahony, J Smarrelli Jr 1980 New forms and new characteristics of higher plant nitrate reductases. *In* GA Akoyunoglou, ed, Proceedings 5th International Congress on Photosynthesis, In press
5. Campbell WH, J Smarrelli Jr 1978 Purification and kinetics of higher plant NADH:nitrate reductase. Plant Physiol 61: 611–616
6. Funkhouser EA, CS Ramadoss 1980 Synthesis of nitrate reductase in *Chlorella*. Plant Physiol 65: 944–948
7. Giri L, CS Ramadoss 1979 Physical studies on assimilatory nitrate reductase from *Chlorella vulgaris*. J Biol Chem 254: 11703–11712
8. Graf L, BA Notton, EJ Hewitt 1975 Serological estimation of spinach nitrate reductase. Phytochemistry 14: 1241–1243
9. Gray JC, RGO Kekwick 1974 An immunological investigation of the structure and function of ribulose 1,5 bisphosphate carboxylase. Eur J Biochem 44: 481–489
10. Hewitt EJ 1975 Assimilatory nitrate-nitrite reduction. Annu Rev Plant Physiol 26: 73–100
11. Hunter WM 1973 Radioimmunoassay. *In* DM Weir, ed, Handbook of Experimental Immunology, Blackwell Scientific Publications, Oxford, pp 17.1–17.36
12. Johnson JL, HJ Cohen, KV Rajagopalun 1974 Molecular basis of the biological function of molybdenum. J Biol Chem 249: 5046–5055
13. Kuo T, A Kleinhofs, RL Warner 1980 Purification and partial characterization of nitrate reductase from barley leaves. Plant Sci Lett 17: 371–391
14. Lord JM, GA Codd, WDP Stewart 1975 Serological comparison of ribulose-1,5-diphosphate carboxylase from *Euglena gracilis*, *Chlorella fusca*, and several blue-green algae. Plant Sci Lett 4: 377–383
15. Notton BA, L Graf, EJ Hewitt, RC Povey 1974 The role of molybdenum in the synthesis of nitrate reductase in cauliflower (*Brassica oleracea* L. var Botrystis L.) and spinach (*Spinacia oleracea* L.). Biochim Biophys Acta 364: 45–58
16. Notton BA, EJ Hewitt 1979 Structure and properties of higher plant nitrate reductase, especially *Spinacia oleracea*. *In* EJ Hewitt, CV Cutting, eds, Nitrogen Assimilation of Plants, Academic Press, New York, pp 227–244
17. Orihuel-Iranzo B, WH Campbell 1980 Pyridine nucleotide specificity and kinetics of soybean leaf NAD(P)H:nitrate reductase. *In* GA Akoyunoglou, ed, Proceedings 5th International Congress on Photosynthesis, In press
18. Ouchterlony O, LA Nilsson 1973 Immunodiffusion and immunoelectrophoresis. *In* DM Weir, ed, Handbook of Experimental Immunology, Blackwell Scientific Publications, Oxford, pp 19.1–19.34
19. Pan S-S, A Nason 1978 Purification and Characterization of homogeneous assimilatory reduced nicotinamide adenine dinucleotide phosphate-nitrate reductase from *Neurospora crassa*. Biochim Biophys Acta 523: 297–313
20. Pateman JA, DJ Cove, BM Rever, DB Roberts 1964 A common cofactor for nitrate reductase and xanthine dehydrogenase which also regulates the synthesis of nitrate reductase. Nature 201: 58–60
21. Small IS, JL Wray 1980 NADH nitrate reductase and related NADH cytochrome c reductase species in barley. Phytochemistry 19: 388–394.
22. Smarrelli J, WH Campbell 1979 NADH dehydrogenase activity of higher plant nitrate reductase (NADH). Plant Sci Lett 16: 139–147
23. Solomonson LP, GH Lorimer, RL Hall, R Burchers, J Leggett-Bailey 1975 Reduced nicotinamide adenine dinucleotide-nitrate reductase of *Chlorella vulgaris*. J Biol Chem 250: 4120–4127
24. White TJ, IM Ibrahami, AC Wilson 1978 Evolutionary substitution and the antigenic structure of globular proteins. Nature 274: 92–94