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EFFECT OF PROTEOLYSIS ON PARTIAL ACTIVITIES AND IMMUNOLOGICAL REACTIVITY OF SPINACH NITRATE REDUCTASE

BRIAN A. NOTTON, ROGER J. FIDO, PETER N. WHITFORD and MICHAEL J. BARBER*

Department of Agricultural Sciences, University of Bristol, AFRC Institute of Arable Crops Research, Research Station, Long Ashton, Bristol BS18 9AF, U.K.; *Department of Biochemistry and Molecular Biology, College of Medicine, University of South Florida, Tampa, FL 33612, U.S.A.

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Key Word Index—*Spinacea oleracea*; Chenopodiaceae; spinach; nitrate reductase; proteolysis; active domains; monoclonal antibodies.

Abstract—Treatment of spinach (*Spinacea oleracea* cv. Noorman) NADH: nitrate reductase (NR), a complex flavohaemomolybdoprotein, with either *Staphylococcus aureus* V8 protease or a naturally occurring protease from corn, resulted in the loss of NADH-cytochrome *c* reductase (NADH:CR) and NADH:dichlorophenolindophenol reductase (NADH:DR) activities but not NADH:ferricyanide reductase (NADH:FR) activity, with no corresponding loss of nitrate-utilising activities. Similar results were obtained for *Chlorella vulgaris* NR. Limited proteolysis of the purified spinach enzyme with V8 protease, followed by FPLC, resulted in the separation of two active protein fragments, a dimer of M_r *ca* 150 000 retaining reduced-methyl viologen (MV:NR), -flavin mononucleotide ($FMNH_2$-NR) and -bromophenol blue (BPB-NR) activities and a monomer of *ca* 30 000 M_r retaining NADH:FR activity. The dimer was recognized immunologically by monoclonal antibodies MAC 74 and MAC 77. Corresponding fragments obtained by proteolysis of *Chorella* NR were *ca* 260 000 M_r and *ca* 30 000 M_r, respectively. These results indicate the presence of a protease-sensitive region that connects the flavin- and haem/Mo-containing domains of spinach NR, analogous to the domain structure established for the closely related NR from *Chlorella*, necessary for the transfer of electrons from NADH to either CR or DR.

INTRODUCTION

Higher plant assimilatory nitrate reductase (NR) (EC 1.6.6.1) is a homodimer of subunits (M_r 110–120 000), each of which contains a FAD, *b*-type cytochrome (b_{557}) and Mo-pterin centre. NADH initially donates electrons to the flavin site followed by electron transfer to the haem and egress, to nitrate, via the Mo site. In addition to the full NADH:NR activity, the enzyme catalyses a number of partial activities, utilising various artificial electron donors and acceptors, that may be classified as either NADH dehydrogenases or nitrate-reducing activities and involve one or more of the enzymes cofactors [1–3].

Incubation of NR from *Chlorella vulgaris* with either V8 protease or corn inactivator proteinase (CIP) resulted in a loss of NADH:NR and NADH:CR activities but no loss of either NADH:FR or MV:NR activities. These partial activities were shown to reside in two different fragments, a monomer of *ca* 30 000 M_r which contained FAD and retained NADH:FR activity and a tetramer of 260 000 M_r which contained haem and Mo and retained MV:NR activity [4].

Proteolysis by CIP of NR from leaves of corn (*Zea mays*), inhibited both NADH:CR and MV:NR activities, the former being much more sensitive to the protease. The CIP also increased the mobility, on native PAGE, of an NR-derived protein which displayed MV:NR activity and cleaved the 116 000 M_r subunit of NR, initially to yield fragments of 84 000 and 80 000. Limited proteolysis of *Chlorella* NR with CIP resulted in fragments of M_rs 68 000 and 28 000 while longer incubation times resulted in more general proteolysis [5].

Products of V8 proteolysis of spinach (*Spinacea oleracea*) NR have been separated by native PAGE and identified by their MV:NR and NADH:FR activities. Subsequent SDS-PAGE of these fragments revealed respectively, multiple polypeptides of M_rs from 50 000 to 80 000 (mostly occurring at M_rs of 50 000 and 70 000) and a single peptide of M_r 28 000 [6]. Trypsin proteolysis of spinach NR has been used [7] to produce a 45 000 M_r fragment displaying NADH:FR and NADH:CR activities with both FAD and haem groups and subsequent V8 proteolysis to split this 45 000 M_r fragment into 28 000 and 14 000 M_r fragments which respectively contained the FAD and haem groups. V8 proteolysis of the native spinach NR produced two fragments of 170 000 and 28 000 M_r retaining respectively $FMNH_2$-(MV):NR and NADH:FR activities [7].

We have utilised limited proteolysis, using both V8 protease and CIP, to examine the effect on the overall activity and six partial activities of purified NR from spinach and *Chlorella*. We have also prepared and characterized active fragments of spinach NR obtained after V8 proteolysis with respect to their residual activities and their ability to recognise monoclonal antibodies (McAbs) which inhibit nitrate reducing activities.

RESULTS

Time course of proteolysis

The effect of limited proteolysis of purified spinach NR on the full, NADH:NR, and partial, NADH:FR, NADH:CR and MV:NR activities are shown in Fig. 1A.

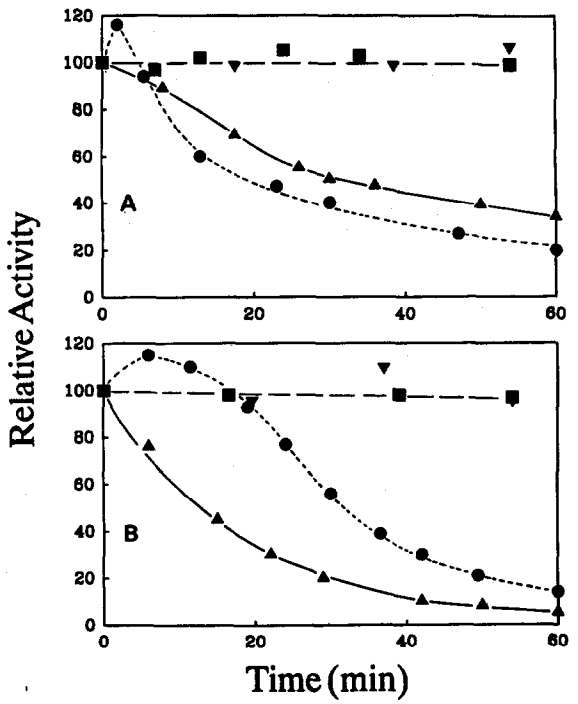

Fig. 1. Time course of proteolysis of NR by *S. aureus* V8 protease. A. Spinach NR (15 μg) was incubated with V8 protease (400 ng) in 50 mM MOPS buffer pH 7 containing 100 μM EDTA and 5 μM FAD at 27° for the indicated times. B. *Chlorella* NR (6 μg) was incubated with V8 protease (170 ng) in 50 mM MOPS buffer pH 7 containing 100 μM EDTA and 5 μM FAD at 27° for the indicated times. Aliquots were withdrawn and assayed for NADH:NR (●), NADH:CR (▲), NADH:FR (▼) and MV:NR (■) activities.

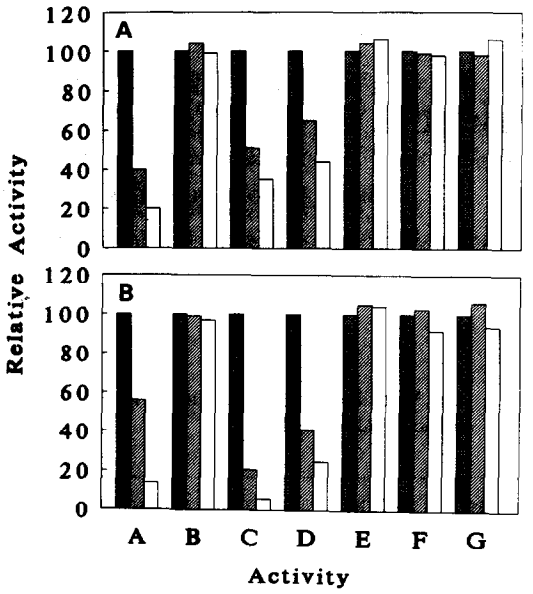

Fig. 2. Effect of *S. aureus* V8 protease on full and partial activities. A Spinach NR (15 μg) was incubated with V8 protease (400 ng) in 50 mM MOPS buffer pH 7 containing 100 μM EDTA and 5 μM FAD at 27° for the indicated times. B. *Chlorella* NR (6 μg) was incubated with V8 protease (170 ng) in 50 mM MOPS buffer pH 7 containing 100 μM EDTA and 5 μM FAD at 27° for the indicated times. Aliquots were withdrawn and assayed for full and partial activities. ■, 0 min; ▨, 30 min; □, 60 min. A, NADH:NR; B, NADH:FR; C, NADH:CR; D, NADH:DR; E, BPB:NR; F, $FMNH_2$:NR; G, MV:NR. Control samples incubated in the absence of protease showed no loss of activity.

Exposure of the enzyme to a limited amount of V8 protease was found to decrease NADH:NR and NADH:CR activities at comparable rates, while the NADH:FR and MV:NR activities remained unaffected. Following one hour of proteolysis, NADH:NR and NADH:CR activities were respectively 20 and 35% of initial values. Brief exposure of the enzyme to the protease resulted in a slight stimulation of NADH:NR activity. Similar results were obtained using the purified *Chlorella* NR (Fig. 1B), although the initial stimulation of NADH:NR activity was more prolonged and the NADH:CR activity decreased at a faster rate than the NADH:NR activity. Following one hour of proteolysis, the NADH:NR and NADH:CR activities had decreased to 14 and 6%, respectively, of initial activities. The overall inactivation pattern was the same for both the spinach and *Chlorella* NR.

Effect of proteases on all NR activities

The effect of treatment of purified spinach and *Chlorella* NRs with V8 protease on the overall NADH:NR activity and six partial activities expressed by the enzymes are shown in Fig. 2 (A and B). For spinach NR, proteolysis for one hour resulted in loss of NADH:NR (80%), NADH:CR (65%) and NADH:DR (56%) activities. For *Chlorella* NR, corresponding losses were 86, 94 and 75%, respectively. With NRs of both species there was no significant loss of either NADH:FR, $FMNH_2$:NR, BPB:NR or MV:NR activities. Treatment of the two NRs with CIP affected the various activities similarly to that observed with V8 protease treatment (Fig. 3A and B). For spinach NR, one hour CIP-proteolysis resulted in loss of NADH:NR (81%), NADH:CR (68%) and NADH:DR (63%) activities and for *Chlorella* NR corresponding losses were 78, 99 and 83, respectively. The action of CIP also caused some loss of nitrate-utilising activities, particularly of spinach NR.

Separation of active domains.

Spinach NR and *Chlorella* NR were both purified by fast protein liquid chromatography (FPLC) on Superose 6 (Pharmacia) to a single chromatographic peak of NADH:NR activity. The enzymes were then subjected to limited proteolysis with V8 protease until less than 10% of the original NADH:NR activity remained. Further protease activity was inhibited with PMSF and the digests chromatographed by FPLC on a calibrated Superose 12 column. Analysis revealed the presence of two activity peaks (Fig. 4A and B). The first, corresponding to a M_r of *ca* 150000 for spinach and 260000 for *Chlorella*, retained MV:NR, $FMNH_2$:NR and BPB:NR activities and the second, corresponding to a M_r of *ca* 30000 for both spinach and *Chlorella* retained only NADH:FR activity.

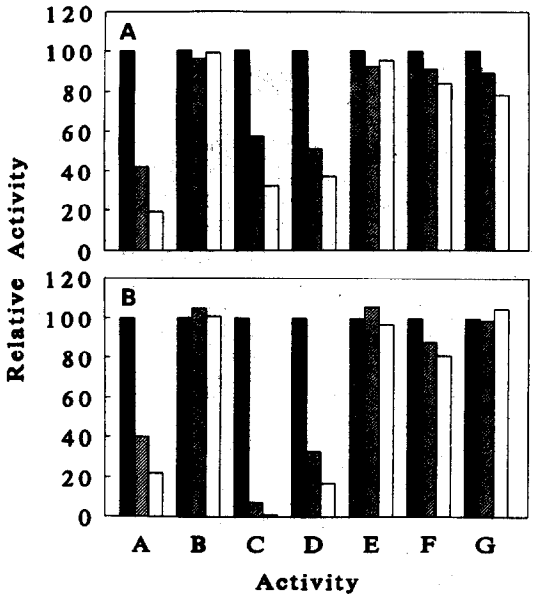

Fig. 3. Effect of CIP on full and partial activities. A. Spinach NR (15 μg) was incubated with CIP (1 μg) in 50 mM MOPS buffer pH 7 containing 100 μM EDTA and 5 μM FAD at 27° for the indicated times. B. *Chlorella* NR (25 μg) was incubated with CIP (1 μg) in 50 mM MOPS buffer pH 7 containing 100 μM EDTA and 5 μM FAD at 27° for the indicated times. Aliquots were withdrawn and assayed for full and partial activities. ▩, 0 min; ▨, 30 min; □, 60 min. A, NADH:NR; B, NADH:FR; C, NADH:CR, D, NADH:DR; E, BPB:NR; F, $FMNH_2$:NR; G, MV:NR; Control samples incubated in the absence of protease showed no loss of activity.

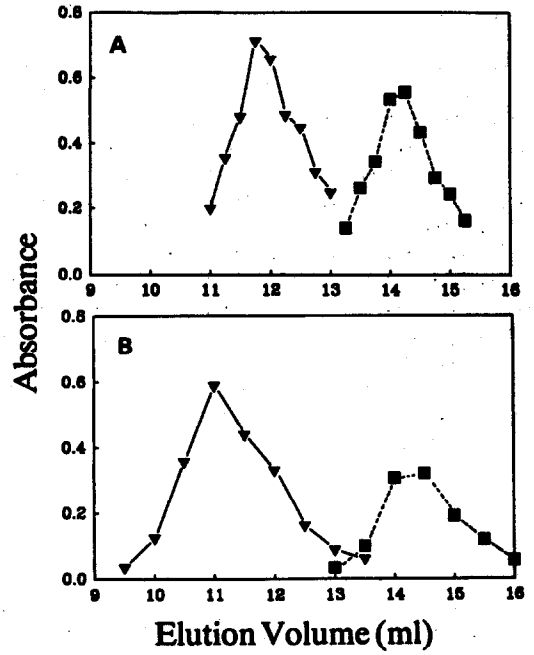

Fig. 4. Separation of products of limited V8 proteolysis of NR. A. Spinach NR (6 μg) was incubated with V8 protease (1 μg) in 50 mM MOPS buffer pH 7 containing 100 μM EDTA and 5 μM FAD at 27° for 10 min, by which time the NADH:CR activity had decreased to 5% of the initial control activity whereas the NADH:FR activity remained unchanged. B. *Chlorella* NR (100 μg) was incubated with V8 protease (15 μg) in 50 mM MOPS buffer pH 7 containing 100 μM EDTA and 5 μM FAD at 27° for 60 min, by which time the NADH:CR activity had decreased to 15% of the initial control activity whereas the NADH:FR activity remained unchanged. Proteolysis was terminated by the addition of PMSF (0.4 mM) and the products separated by FPLC using a Superose 12 column. Fragments were identified by retention of MV:NR (▼) and NADH:FR (■) activities.

Antigenicity of domains against monoclonal antibodies

After proteolysis and separation of active domains by FPLC, the fractions containing MV:NR and NADH:FR activities were tested for their ability to bind various McAbs raised against spinach NR [8]. MAC 74 and MAC 77, which have the ability to inhibit nitrate-utilizing reactions of NR [8], showed positive dot-blots only with the MV:NR fraction and inhibited all associated activities, while the NADH:FR fraction was neither immunologically recognised nor was its activity inhibited. If either of the antibodies were added to the proteolysed-NR and the mixture then subjected to FPLC, NADH:FR activity was still detected in the fractions corresponding to a M_r of *ca* 30 000, as in Fig. 4A, while the fractions corresponding to a M_r of *ca* 150 000 contained no enzyme activities. Addition of either of the antibodies to the native NR, followed by FPLC, resulted in the NR-associated NADH:FR activity being excluded in the fraction (8.5 ml) corresponding to a M_r in excess of 300 000 whereas native NR occurs in the fraction (10.5 ml), equivalent to a M_r of *ca* 200 000.

Products of V8 proteolysis of spinach NR

The products of limited proteolysis were examined by SDS-PAGE. Purified native NR from spinach was resolved into a doublet of M_rs of 115 000 and 105 000 and a component at 50 000. This latter material is commonly observed after SDS-PAGE of purified spinach NR, is antigenically positive against polyclonal anti-NR and is thought to be a proteolytically nicked subunit of the NR, which remains tightly associated with the native enzyme until disruption by SDS [9]. Inactivation by V8 protease decreased the intensity of the initial doublet in a time-dependent manner and resulted in the appearance of a doublet of M_rs of 85 000 and 75 000, the latter being predominant and a singlet of M_r 28 000 (Fig. 5). Proteolysis of the 105 000 M_r protein band, previously excised and electroeluted from a SDS-PAGE gel, revealed only two protein bands of M_rs *ca* 75 000 and 30 000 on subsequent resolution by SDS-PAGE (data not shown).

DISCUSSION

The preceeding results demonstrate that limited proteolysis of spinach NR, using either V8 protease or CIP, generates protein fragments that retain only some of the partial activities exhibited by the native enzymes. The initial increase in NADH:NR activity exhibited by both spinach NR and *Chlorella* NR, on treatment with V8 protease, was followed by a concommitent loss of both NADH:NR and NADH:CR activities. This indicated an

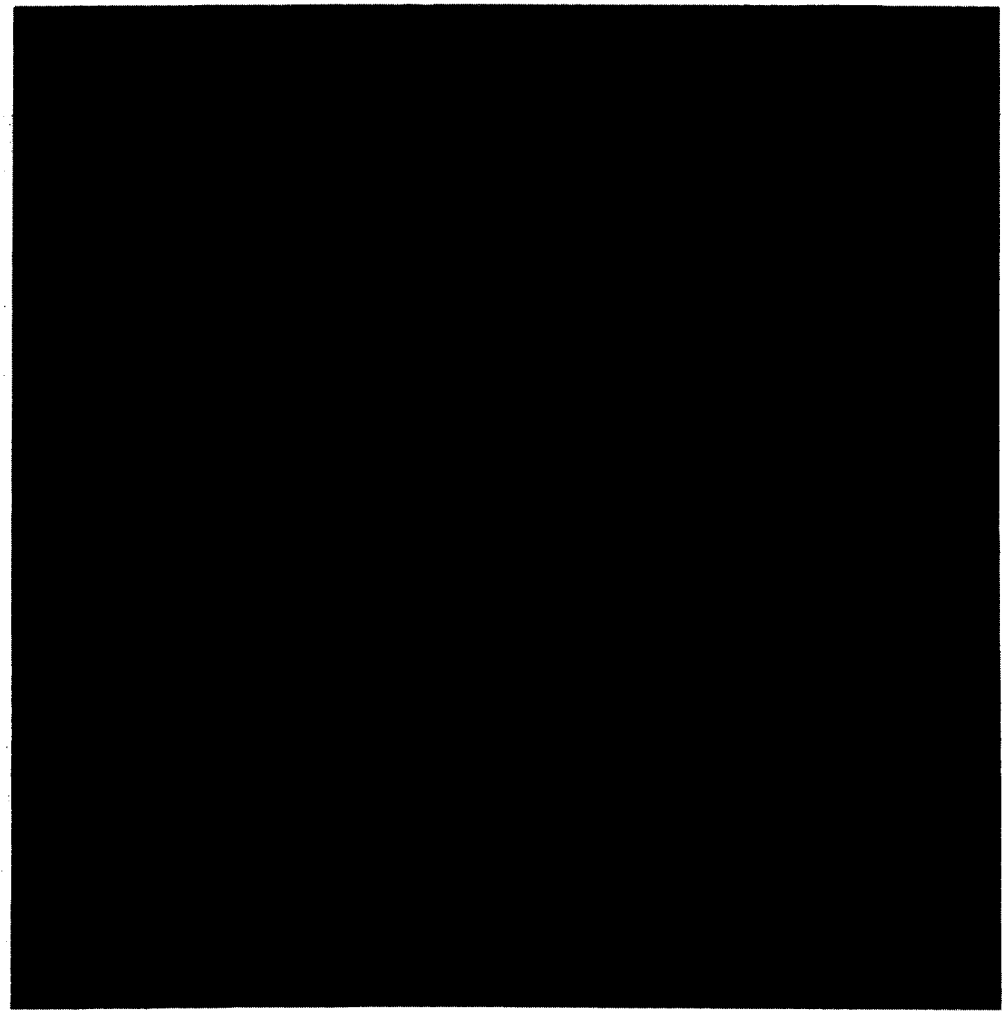

Fig. 5. SDS-PAGE of spinach NR progressively inactivated by V8 protease. Spinach NR (12 μg) was incubated with V8 protease (1 μg). Aliquots were removed after 0, 5, 10 and 20 min and the proteolysis terminated with PMSF (0.4 mM). Standard proteins were run as reference M_rs.

absence of non-NR NADH:CR species, which were suspected to be present in the corn NR preparations used by Poulle *et al.* [5] and which gave rise to residual NADH:CR activity after complete inactivation of the NADH:NR activity by CIP.

The result of V8 proteolysis on all activities expressed by both spinach NR and *Chlorella* NR showed that, in addition to the effects previously observed [4, 7], NADH:DR activity was inhibited and BPB:NR activity was unaffected. The inhibition of NADH:DR activity suggests that this dehydrogenase function is dependent on the presence of the haem group in the same way as NADH:CR activity. This confirms the early observation [10], which showed the reoxidation of the NADH-reduced haem of spinach NR by addition of excess DCPIP. The stability of the BPB:NR activity supports the conclusion that reduced BPB acts in a manner similar to reduced MV although the exact binding site may not be the same [11].

The results obtained using CIP were very similar to those obtained using V8 protease. However, there was a small loss of $FMNH_2$:NR and MV:NR activities of the spinach NR which was much less than that previously observed with corn NR [5]. The nitrate-reducing activities of the *Chlorella* NR were less sensitive to CIP, supporting previous work [4] and suggesting a minor structural difference in NR between algae and higher plants. For all activities examined, similar results were obtained for the spinach and *Chlorella* enzymes suggesting that a conserved site in both proteins is susceptible to the action of both of these proteases.

Separation of the functional domains obtained after V8 proteolysis, using FPLC on a calibrated Superose 12 column, showed that, while the NADH:FR activity resided in a fragment of M_r *ca* 30000 with both spinach NR and *Chlorella* NR, the MV:NR was associated with a fragment of M_r *ca* 150000 with spinach NR, while the same activity was in a fragment of M_r *ca* 260000 with the *Chorella* NR. This confirmed previous results obtained with *Chlorella* NR, when the separation was achieved using a Sephacryl S-300 column [4], and is similar to the results obtained with spinach using HPLC on TSK G3000SW [7]. By analogy with the extensive work on the nature of the fragments obtained by proteolysis of the

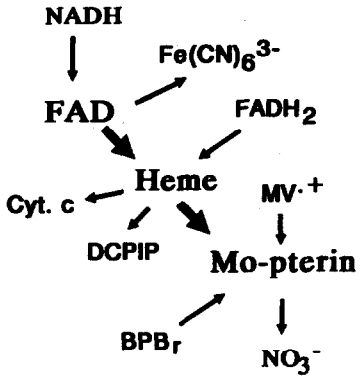

Fig. 6. Model of spinach NR showing sites of interaction of various electron donors and acceptors.

Chlorella NR [4, 5] and spinach [6, 7], the fragments of M_r *ca* 30000 and *ca* 150000 contain FAD and haem/Mo, respectively.

The previously observed inhibitory effect of MAC 74 and MAC 77 on the nitrate-dependent activities of NR and the postulation [8] that this inhibition was due to the binding of the McAbs to the haem/Mo domain of the enzyme, was confirmed. The inability of the McAbs to affect either the size or the NADH:FR activity of the isolated small domain, together with the change in M_r of the associated NADH:FR activity of the holoenzyme, as a result of the formation of an NR-McAb complex, indicated that both of these McAbs did indeed bind to the haem/Mo domain of NR. This is in direct contrast to the apparently contradictory results obtained by Sueyoshi *et al.* [6], with their McAbs obtained against spinach NR. They reported that their two McAbs inhibited the NADH:CR, $FMNH_2$:NR and MV:NR activities of the holoenzyme but not the NADH:FR activity. However, the PAGE-isolated domains, immunoblotted following SDS-PAGE, showed exclusive McAb-binding to the small fragment. Their results indicate that their McAbs bind to the small domain, without inhibiting its NADH:FR activity, but cause inhibition of the nitrate-dependent activities associated with the large domain. They suggest that this may be due to steric hinderance or conformational change of the enzyme by McAb binding.

Subunit size of the fragments from V8 proteolysis of the spinach NR, was confirmed using SDS-PAGE. The fragment of M_r *ca* 30000 was monomeric, as found for the *Chlorella* [4] and spinach NR [7], whereas the fragment of M_r *ca* 150000 was dimeric [7], in contrast to the tetrameric fragment of M_r 260000 previously shown for *Chlorella* [4].

These results can be summarized in terms of a structure–function model for spinach NADH:NR, shown in Fig. 6. While only one of the subunits is shown for the homodimer enzyme, the results of the limited proteolysis experiments show that the NADH-binding site and FAD comprise part of the fragment of M_r *ca* 30000 and thus retains NADH:FR activity, whereas the haem and Mo centres are located within the fragment of M_r *ca* 150000 and thus retains $FMNH_2$-NR, BPB:NR and MV:NR activities. Cleavage of the protein at the V8 and CIP-sensitive sites effectively prevents electron transfer from reduced FAD to haem and destroys NADH:CR and NADH:DR activities.

EXPERIMENTAL

Sources of nitrate reductase. Spinach NR was purified using an immunopurification procedure [12] followed by FPLC on Superose 6 (Pharmacia Ltd). Purified enzyme exhibited an NADH:NR specific activity of 1.5 μkat/mg protein. *Chlorella* NR was purified by affinity chromatography [13] also followed by FPLC on Superose 6. NADH:NR specific activity of the enzyme was 1.53 μkat/mg protein. Protein was determined using the Bio-Rad dye binding assay (microassay procedure) with BSA as reference protein.

Enzyme assays. NADH:NR activity was measured at 340 nm as nitrate-dependent (4 mM) oxidation of NADH (100 μM) [14]. NADH:FR activity was measured at 340 nm as ferricyanide-dependent (2 mM) oxidation of NADH (100 μM) [14]. NADH:CR activity was measured at 550 nm as the NADH-dependent (100 μM) reduction of cytochrome *c* (44 μmM) [14]. NADH:DR was measured at 620 nm as the NADH-dependent (50 μM) reduction of DCPIP (50 μM) [15]. $FMNH_2$:NR, [16] BPB:NR [11] and MV:NR [17] activities were measured by the reduction of nitrate to nitrite using dithionite-reduced substrate.

Inactivation of NRs by proteases. Purified NRs were incubated at 27° in 50 mM MOPS buffer, pH 7.0 containing 0.1 mM EDTA and 5 μM FAD, in the presence or absence of the selected protease. Aliquots were withdrawn at selected time intervals and both full and partial activities remaining were determined.

Isolation of enzyme fragments. Purified NRs from spinach and *Chlorella* were incubated with V8 protease at 27° in 50 mM MOPS buffer pH 7.0, containing 0.1 mM EDTA and 5 μM FAD, until greater than 90% of the initial NADH:NR activity had been abolished, following which PMSF was added (1 mM final concn) and the digest chromatographed by FPLC using a Superose 12 column. Fractions were assayed for MV:NR and NADH:FR activities.

Electrophoresis. SDS-PAGE was done using the method of ref. [18] with 10% separating gel and 3.75% stacking gel. The proteins were heated to 90° for 5 min in the presence of SDS and 2-mercaptoethanol before running.

Immunoblotting. Immunological recognition by McAbs of FPLC-separated protein fragments obtained, following V8 proteolysis of spinach NR, used the dot blot technique described previously [19].

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