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Escherichia coli Nitrate Reductase Subunit A: Its Role as the Catalytic Site and Evidence for Its Modification

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Subunits A and B were isolated from purified nitrate reductase by preparative electrophoresis in low levels of sodium dodecyl sulfate. Nonheme iron and low levels of molybdenum were associated with isolated subunit A but not with isolated subunit B. After dialysis against a source of molybdenum cofactor, subunit A regained tightly bound molybdenum and concomitantly regained enzyme activity and reactivity with anti-nitrate reductase antiserum. Subunit B neither bound cofactor nor regained activity or reactivity with antiserum. These data indicate that subunit A contains the active site of the enzyme. Subunit A was also found to be modified posttranslationally in a similar fashion as is subunit B. This was determined by comparison of partial proteolytic digests and amino acid analyses of A subunits from precursor and membrane-bound forms of nitrate reductase.

Nitrate reductase (EC 1.7.99.4) is a membrane-bound anaerobic respiratory enzyme in *Escherichia coli* which is repressed by O_2 and induced by NO_3^-. It was classified as a type A enzyme by Pichinoty and Picchaud (28). The enzyme complex catalyzes the flow of electrons to the final electron acceptor, nitrate (12). It is composed of three subunits, A, B, and C. Subunit C (molecular weight [MW], 20,000) is a *b*-type cytochrome (22) which mediates the flow of electrons from formate dehydrogenase to the catalytic site of nitrate reductase. It is considered to be required for the functional assembly of the enzyme into the membrane and may play a role in regulating the biosynthesis of the enzyme (23). Subunit B (MW, 60,000) has been postulated to be involved in membrane attachment (2). It is synthesized in the cytoplasm in a form which is different from that found in the membrane. The modification of the cytoplasmic precursor form of subunit B (B′) is an event which takes place after the translocation of enzyme into the membrane, although both forms may be present in the membranes of mutant strains lacking functional nitrate reductase activity (24).

Subunit A is the largest (MW, 142,000) subunit of nitrate reductase; however, relatively little is known about it. It has been proposed (2) to be the catalytic subunit, since treatment of the enzyme complex with low levels of trypsin did not decrease the enzyme activity, although subunit B was degraded to a smaller peptide (MW, 43,000). Subunit A, in this case, appeared to be unaffected by this treatment (2). More recently, limited iodination has been reported to inhibit activity when only subunit A is iodinated (10). To determine if the catalytic site resides only with subunit A, we chose the following approaches. First, we sought to identify which subunit was associated with the molybdenum cofactor. This cofactor has been recognized to be essential for nitrate reduction (7, 30, 31). Second, we dissected the enzyme into its individual subunits and determined which isolated subunit contained the nitrate reductase activity.

Subunit A, like subunit B, has been shown to be synthesized in the cytoplasm and then translocated to the membrane (11); however, no precursor form of subunit A has been identified by pulse-chase techniques (24). Thus, we set out to determine if the precursor form of subunit A found in the cytoplasm was different from that found in the membrane-bound enzyme complex.

MATERIALS AND METHODS

Materials. Dithiothreitol (DTT), glutathione (GSH), mercaptoethanol, and Triton X-100 were obtained from Sigma Chemical Co., St. Louis, Mo., and *Staphylococcus aureus* V8 protease was obtained from Miles Laboratories, Inc., Elkhart, Ind. Guanidine hydrochloride (GHCl) was purchased from Pierce Chemical Co., Rockford, Ill. MW standards were obtained from Bio-Rad Laboratories, Richmond, Calif.

Growth conditions. *E. coli* MC4100 was grown at 37°C under anaerobic conditions in minimal salts medi-

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um containing amino acids and vitamins, with glucose as the carbon source (11). Aerobic cells were grown at 37°C with vigorous shaking in medium containing only minimal salts (no NO_3) plus 1 mM sodium molybdate, with succinate as the carbon source. Both aerobic and anaerobic cells were harvested at the late logarithmic phase, washed twice with 0.1 M Tris-hydrochloride (pH 7.2), and converted to spheroplasts (32). These were frozen and used as needed. Strain MC4100 was labeled with 3 to 5 μCi of $H_2{}^{35}SO_4$ per ml (Amersham Corp., Arlington Heights, Ill.) in medium lacking cysteine and methionine and in which all the sulfate salts were replaced with chlorides. Na_2SO_3 (13 μg/ml) was added to the medium as a minimal source of sulfur.

A *nit-1*-containing *Neurospora crassa* mutant strain was kindly supplied by Reginald Garrett, Biology Department, University of Virginia, Charlottesville. It was grown in liquid culture with Fries basal medium as described by Garrett (5).

Isolation of subunits A and B. Nitrate reductase was purified as described elsewhere (G. R. Chaudhry and C. H. MacGregor, J. Biol. Chem., in press). The purified enzyme (5 to 10 mg/ml) was diluted with 0.25 M Tris-hydrochloride (pH 6.8)–5% DTT–2% mercaptoethanol–0.5% sodium dodecyl sulfate (SDS)–1 M GHCl, incubated at room temperature for 30 min, and then passed through a small column of Sephadex G-25 equilibrated with the above-described buffer lacking GHCl.

The eluted material was adjusted to contain 10% glycerol and immediately loaded onto a preparative gel electrophoresis column. The purified enzyme migrated as three separate bands. The leading light-yellow-colored band was that of cytochrome *b* (subunit C); it was followed by a colorless band (subunit B) and a brownish band (subunit A). Subunits A and B were collected separately, and their purity was routinely checked by SDS-polyacrylamide gel electrophoresis.

Preparation of crude cytoplasm for reconstitution experiments. The spheroplasts from aerobically grown cells were lysed in 5 mM Tris-hydrochloride (pH 7.4) containing 1 mM GSH, 1 mM DTT, 0.5 mM $MgCl_2$, RNase, and DNase. The lysate was centrifuged at 15,000 × *g* for 30 min, and the supernatant was used for reconstitution studies after the Tris-hydrochloride concentration was raised to 50 mM. The presence of molybdenum cofactor in the supernatant was tested by a complementation assay as follows. A crude extract of a *nit-1*-containing *N. crassa* mutant strain in 50 mM Tris-hydrochloride (pH 7.2)–1 mM EDTA–20 mM $NaMoO_4$ was incubated with the crude cytoplasmic preparations (2:1) for 15 min. This mixture was then assayed for NADPH-nitrate reductase activity by the method of Garrett and Nason (6).

Reconstitution of nitrate reductase activity. Each isolated subunit of nitrate reductase (1 to 2 mg of A or B) was treated as follows. Immediately after isolation, each sample was adjusted to contain 2% Triton X-100–5 mM DTT–1 mM GSH–0.5 M GHCl and then dialyzed twice against the cytoplasm from aerobically grown *E. coli* cells for a period of 3 to 4 h. The dialysis bag was then transferred to buffer containing 50 mM Tris-hydrochloride (pH 7.4), 1 mM EDTA, 1 mM GSH, 0.5 mM DTT, 1% mercaptoethanol, and 0.1% Triton X-100. After 4 h the dialysis buffer was replaced with the same buffer except that mercaptoethanol was omitted, and the fraction was dialyzed for several more hours. This fraction was tested for nitrate reductase activity (see Fig. 1).

Partial proteolysis. To isolate ^{35}S-labeled subunit A, we purified or immunoprecipitated (24) the enzyme from continuously labeled cells and subjected it to SDS-polyacrylamide slab gel electrophoresis. After electrophoresis the gels were dried, and the location of subunit A on the gels was determined by radioautography. This band was then cut and eluted in 50 mM NH_4HCO_3 buffer containing 0.1% SDS. The eluted fractions were lyophilyzed and passed through a small column of Sephadex G-25 equilibrated with 0.1 M Tris-hydrochloride (pH 7.2).

Partial proteolysis of isolated subunit A was carried out in 100 mM Tris-hydrochloride (pH 7.2) containing 0.05% SDS. The samples were treated with 3 μg of trypsin or papain or 10 μg of V8 protease per ml. The reaction mixture for trypsin also included 10 mM $CaCl_2$. At the end of the reaction the samples were dissolved in electrophoresis sample buffer containing 150 mM Tris-hydrochloride (pH 6.8), 2% SDS, 5% mercaptoethanol, 10% glycerol, and 1 mM EDTA and boiled for 3 min. All samples were analyzed by gel electrophoresis on a 5 to 20% gradient of acrylamide as described below.

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed by the method of Lugtenberg et al. (19). Samples were prepared by heating at 100°C in sample buffer for 2 min. Protein bands were detected either by staining with Coomassie blue (4) or by radioautography (24).

Preparative electrophoresis was carried out by a method described elsewhere (Chaudhry and MacGregor, in press).

Absorption spectra. Absorption spectra were recorded at room temperature with either a Beckman Acta CV spectrophotometer or a Cary 219 spectrophotometer. Sample cuvettes with a 1-cm light path were used.

Assays. Nitrate reductase activity was assayed at room temperature with methyl viologen as the electron donor (25). One unit of activity is defined as the production of 1 μmol of NO_2 per min. For the isolated subunits nitrate reductase activity was estimated in the presence of 1 mM Mo, as was the case in reconstitution studies.

Metal content was determined by atomic absorption spectroscopy (13). Amino acid analyses were performed with a Beckman amino acid analyzer (model 121 M). The samples were hydrolyzed for 24, 48, and 72 h in vacuo at 110°C in constant boiling HCl (26). Cysteine was estimated as cysteic acid after performic acid oxidation (14). Tryptophan was estimated by measuring the absorbance at 280 nm (9). Protein determinations were made by the method of Lowry et al. (18).

RESULTS

Reconstitution of nitrate reductase activity. Amy and Rajagopalan (1) showed that the cytoplasmic fraction of aerobically grown *E. coli* cells contains a dialyzable molybdenum cofactor which can reconstitute nitrate reductase activity in crude extracts of a *Neurospora* mutant lack-

ing NADPH-nitrate reductase activity. We used this unfractionated *E. coli* cytoplasm as a source of molybdenum cofactor. To determine which of the nitrate reductase subunits contained the active site, we attempted to reconstitute the lost enzyme activity in the purified subunits by adding molybdenum cofactor. For reconstitution of nitrate reductase activity the use of low SDS concentrations for the isolation of the individual subunits was crucial. Neither of the subunits (A or B) retained nitrate reductase activity when isolated under normal conditions for polyacrylamide gel electrophoresis in the presence of SDS. However, when the SDS concentration was reduced to 0.5% and when the samples were not boiled before electrophoresis, the dissociation of subunits A and B was incomplete (Fig. 1, lane 2). When samples containing low SDS concentrations were treated with GHCl and incubated for 30 min, not only was the dissociation of subunits A and B complete, but the isolated subunit A also was able to regain nitrate reductase activity upon restoration of molybdenum cofactor (see below). Immediately after being removed from the electrophoresis column, however, purified subunits A and B had little or no activity.

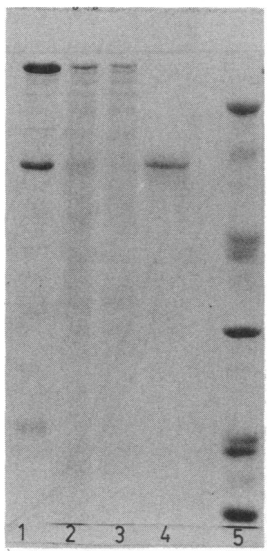

FIG. 1. SDS-polyacrylamide gel analysis of the subunits of nitrate reductase used for reconstitution of activity. (Lane 1) Purified nitrate reductase; (lane 2) subunit A isolated in the presence of 0.5% SDS; (lanes 3 and 4) subunits A and B, respectively, isolated after 0.5% SDS–GHCl treatment; (lane 5) standards phosphorylase B (MW, 92,000), bovine serum albumin (MW, 66,000), ovalbumin (MW, 45,000), carbonic anhydrase (MW, 31,000), soybean trypsin inhibitor (MW, 21,500), and lysozyme (MW, 14,000). All the samples were dissolved in standard sample buffer and heated for 1 min before electrophoresis. The acrylamide concentration was 12.5%.

TABLE 1. Effect of pH on the reconstitution of nitrate-reducing activity in nitrate reductase subunits[a]

Enzyme or subunit	pH	% Activity
Purified enzyme[b]		100
Purified enzyme + SDS + GHCl		85
Isolated subunit A		1.2
Isolated subunit B		0
Subunit A treated for reconstitution	8.0	0
	7.4	29.4
	7.2	21.0
	7.0	9.9
	6.5	0
Subunit B treated for reconstitution	8.0	0
	7.4	0
	7.2	0
	7.0	0
	6.5	0

[a] The isolated subunits A and B were treated for reconstitution at various pHs as described in Materials and Methods. The enzyme activity recovered was compared with purified enzyme activity. All samples were at pH 7.4 at the time of assay. Dialysis of subunit A or B against pH 7.4 buffer containing 10^{-3} M $NaMoO_4$ but no cytoplasmic extract resulted in no increase in enzyme activity above that found in the isolated subunit.

[b] The specific activity of the purified enzyme containing all three subunits was ca. 50 μmol of NO_2 produced per minute per milligram of protein.

After carrying out the procedure described above, we were able to reconstitute nitrate reductase activity in only one of the subunits of the enzyme. Table 1 shows that subunit A regained nitrate reductase activity after incubation with a source of molybdenum cofactor but that subunit B did not.

It should be noted that the cytoplasmic extract used as a source of molybdenum cofactor in reconstitution experiments had no nitrate reductase activity itself. Using antibody to the purified enzyme, we have also shown that no antibody-precipitable nitrate reductase is present in cytoplasmic extracts from aerobic cells (unpublished data).

Analysis of iron and molybdenum. Another way of identifying the catalytic site was to observe the fate of the metal components of nitrate reductase that are considered essential for activity. These are molybdenum and nonheme iron.

Both iron and molybdenum were completely lost during standard SDS-polyacrylamide gel electrophoresis, as was the enzyme activity. When preparative column electrophoresis was carried out as described elsewhere (Chaudhry

TABLE 2. Iron and molybdenum content in nitrate reductase and purified subunits A and B

Enzyme or subunit	Fe[b] per mol	Mo[b] per mol
Pure[a] subunit A	9.3	0.13
Pure subunit A dialyzed against buffer	1.1	0.09
Pure subunit A dialyzed against crude cytoplasm	7.4	0.43
Pure[a] subunit B	0.5	0.06
Pure subunit B dialyzed against buffer	0.3	0.02
Pure subunit B dialyzed against crude cytoplasm	0.35	0.03
Pure enzyme containing subunits A and B[c]	12.0	0.52
Pure enzyme containing subunits A, B, and C	16.0	0.61

[a] Subunits A and B were isolated as described in the text, and metal content was determined before and after treatment for reconstitution.

[b] Iron and molybdenum were estimated on the basis of MWs of 142,000 for subunit A, 60,000 for subunit B, 200,000 for the AB' form, and 240,000 for the ABC form.

[c] This form of the enzyme was purified along with the ABC form (Chaudhry and MacGregor, in press).

and MacGregor, in press), nonheme iron remained associated with subunit A (Table 2), as suggested by the brown color of the purified subunit. Under the same conditions, however, molybdenum was lost. Although the enzyme activity was only slightly affected by dilution into SDS containing buffer, it was almost completely lost, along with the molybdenum, during electrophoresis.

The determinations of iron and molybdenum in the isolated subunits are shown in Table 2. The majority of the iron was found associated with subunit A. Dialysis of isolated subunit A in 100 mM Tris-hydrochloride buffer (pH 7.4) caused the loss of about 90% of the iron; however, when dialysis was performed under reconstitution conditions, iron was retained (Table 2), albeit at only 7.4 atoms per molecule. The amount of iron in subunit B was not significant. This suggests that in the holoenzyme, the nonheme iron is associated with subunit A.

A reduced amount of molybdenum was detected in isolated subunit A. This amount increased significantly from 0.13 to 0.43 mol per mole of subunit A with the restoration of nitrate reductase activity. The amount of molybdenum in subunit B was very small and did not increase during reconstitution experiments.

Absorption spectra. The oxidized absorption spectrum of reconstituted subunit A had a yellow color and a broad absorption peak at 415 nm. The nonheme iron-containing enzyme, hydrogenase, has been shown to exhibit a plateau in this range of the spectrum (15). The color of the fraction was bleached upon reduction with dithionite, with a concomitant change in the spectrum; i.e., the absorption decreased throughout the visible range (Fig. 2). These spectral properties were similar to that of the modified form of nitrate reductase (Chaudhry and MacGregor, in press), which contains only subunits A and B. The absorption spectrum of subunit B had no characteristic features. As expected, the cytochrome *b* peak associated with subunit C (22) was entirely lacking in all the preparations.

Immunoprecipitation of reconstituted subunit A. Since subunit A regained nitrate reductase activity, we presumed that it had refolded to its original or near-original structure. It was therefore likely that this subunit A would react with antibody raised against the AB form of the enzyme (21). Figure 3 shows the results of Ouchterlony analysis of subunit A. Subunit A lacking enzyme activity did not react with antibody even when isolated under milder conditions. However, subunit A was recognized by antibody after regaining nitrate reductase activity. Purified subunit B, on the other hand, did not react with antibody under any conditions.

Partial proteolysis. Posttranslational changes

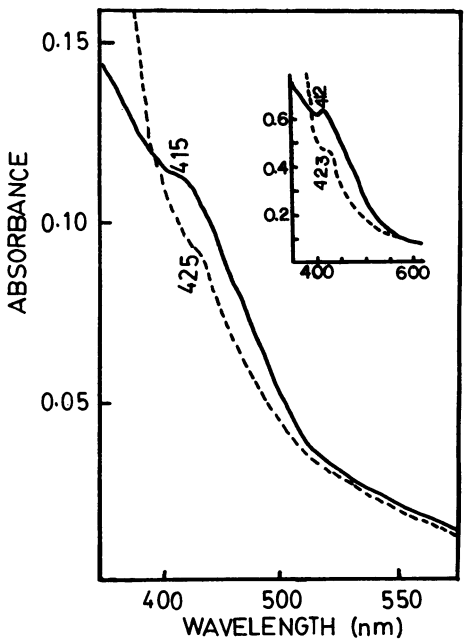

FIG. 2. Visible absorption spectra of reconstituted subunit A of nitrate reductase. The oxidized form (——) was reduced with a few grains of dithionite (- - - -) in buffer containing 50 mM Tris-hydrochloride (pH 7.4), 1 mM GSH, and 1% Triton X-100. The insert shows the absolute oxidized (——) and reduced (- - - -) spectra of modified nitrate reductase.

in subunit B during its biosynthesis have been reported previously (24). The precursor form of subunit B found in the cytoplasm (B') migrates on SDS gels with an apparent MW approximately 2,000 less than that of the completed membrane-bound form of subunit B. Giordano et al. (8) have suggested that subunit A might also be synthesized in a precursor form, but the experiments which demonstrated the precursor form of subunit B (24) identified no MW differences in subunit A. During the purification of nitrate reductase a variable percentage of modified enzyme can be separated from the complete enzyme. This modified nitrate reductase contains subunit B with an altered mobility on SDS gels. All analyses of B subunit from this modified enzyme indicated that it is identical to the B' precursor (G. R. Chaudhry and C. H. MacGregor, J. Biol. Chem., in press). As with the precursor studies (24), no change in mobility on SDS gels is seen in subunit A from the modified enzyme (Chaudhry and MacGregor, in press). To look for posttranslational changes in subunit A, we compared proteolytic fragments of subunit A preparations from several sources. Subunit A was isolated from purified, complete nitrate reductase, from nitrate reductase modified by the activity of an enzyme which changes nitrate reductase during purification (Chaudhry and MacGregor, in press), and from the precursor form of the enzyme obtained by immunoprecipitation from the cytoplasmic fraction (24).

Proteolytic digests of each subunit A preparation were analyzed by SDS-polyacrylamide gel electrophoresis. Figure 4 shows the comparison of proteolytic fragments obtained by partial digestion using V8 protease. It is evident that the modified and cytoplasmic forms of subunit A had all peptides in common, but a few peptides from subunit A isolated from the completed enzyme were different. On the basis of these results we concluded that subunit A from the modified and cytoplasmic forms of nitrate reductase appear to be identical and that both of these forms are different from membrane-bound subunit A. Trypsin and papain yielded a large number of proteolytic fragments, and comparison of these fragments showed the same result (data not shown). A similar analysis of subunit B isolated from these two forms of nitrate reductase demonstrated that the cytoplasmic precursor form of subunit B and modified subunit B were also identical and that they were different from membrane-bound subunit B (Chaudhry and MacGregor, in press).

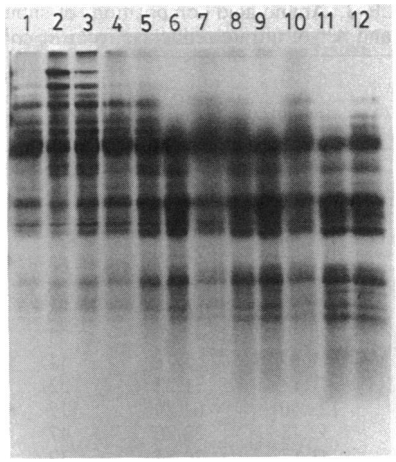

FIG. 4. Comparison on SDS gels of peptide patterns produced by limited digestion of ^{35}S-labeled subunit A isolated from unmodified, purified enzyme, modified enzyme, and cytoplasmic enzyme with V8 protease. The reaction mixture in a total volume of 150 μl contained approximately 3.5×10^5 cpm of subunit A, 100 mM Tris-hydrochloride (pH 7.2), and 0.05% SDS. The digestions were started by the addition of 10 μg of protease per ml. Samples (25 μl) were drawn at intervals of 1, 5, 10, and 30 min, dissolved in SDS sample buffer containing 1 mM EDTA as described in Materials and Methods, and immediately boiled for 3 min. These were run on 15% acrylamide gels in the presence of SDS, fixed in 10% acetic acid, and dried for autoradiography. (Lanes 1, 4, 7, and 10) Subunit A from purified enzyme; (lanes 2, 5, 8, and 11) subunit A from modified enzyme; (lanes 3, 6, 9, and 12) subunit A from precursor enzyme. Incubation times were as follows: lanes 1–3, 1 min; lanes 4–6, 5 min; lanes 7–9, 10 min; and lanes 10–12, 30 min.

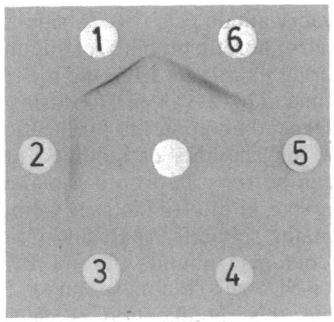

FIG. 3. Double-diffusion immunoprecipitation. Subunits A and B were isolated as described in the text. The center well contained antisera against nitrate reductase. The numbered wells contained the following: (1) unmodified, purified nitrate reductase; (2) nitrate reductase treated with SDS-GHCl; (3) isolated subunit A; (4) isolated subunit B; (5) subunit B after treatment for reconstitution; and (6) subunit A after treatment for reconstitution.

Amino acid analysis. Amino acid analysis of subunit A isolated from purified enzyme and subunit A isolated from modified enzyme (A') (Table 3) indicated that the residues of amino acids were comparable in both cases.

TABLE 3. Amino acid composition[a] of subunits A and A'[b] of nitrate reductase from *E. coli*

Amino acid	A (nmol)	A' (nmol)	No. of residues per mol[c]	
			A	A'
Asp	168.13	87.91	152	153
Thr[d]	100.46	47.96	91	86
Ser[d]	89.57	46.27	81	80
Glu	151.44	75.19	136	130
Pro	74.95	37.02	68	64
Gly	130.65	72.30	118	125
Ala	117.20	60.15	106	104
Val[e]	80.60	45.69	73	79
Met[d]	11.87	5.78	11	10
Ile[e]	57.13	31.23	52	54
Leu[e]	123.60	64.20	112	111
Tyr[d]	52.48	27.76	47	48
Phe	43.17	20.24	39	35
Lys	76.98	45.69	69	79
His	37.99	18.51	34	32
Arg	79.91	39.91	72	69
Cys[f]	7.47	3.75	13	13
Trp[g]	15.01	9.25	17	16

[a] Data are reported as the average of three or more determinations.

[b] Subunit A was isolated from purified cytochrome-containing enzyme, and subunit A' was isolated from modified, purified nitrate reductase (Chaudhry and MacGregor, in press).

[c] Based on an MW of 142,000. The number of hydrophobic residues of subunit A was 478 (37%), and that of subunit A' was 473 (36.7%).

[d] Corrected for destruction by extrapolation to zero time.

[e] Data are reported as the average of 48- and 72-h hydrolyses.

[f] Determined after performic acid oxidation.

[g] Estimated by a spectrophotometric method.

DISCUSSION

This paper provides several lines of evidence indicating that subunit A contains the active site of the enzyme nitrate reductase.

First, in purified isolated subunits, molybdenum and nonheme iron were found associated with subunit A but not with subunit B. Both of these metals have been suggested to be associated with the active site of this enzyme (7, 30, 31). After dialysis with a soluble extract from aerobically grown cells, the molybdenum content of subunit A increased to levels close to those found in the purified enzyme. The fact that this association was specific is indicated by three lines of evidence. There is a concomitant increase in molybdenum content and in enzyme activity, subunit A is precipitated by antibody only after this dialysis, and the reconstituted level of molybdenum is not lost from subunit A after prolonged dialysis in buffer. Subunit B showed no increase in metal content, activity, or reaction with antibody after dialysis with the cytoplasmic extract.

Second, enzyme activity was found (although at a low level) only in purified subunit A and could be reconstituted to 30% of the level found in the purified enzyme. Ramadoss et al. (29) have inserted molybdenum in their demolybdo-nitrate reductase from *Chlorella vulgaris* in vitro; however, *apo*-nitrate reductase preparations from plants required complete molybdenum cofactor for restoration of enzyme activity (27). We could reconstitute nitrate reductase in isolated subunit A by adding molybdenum cofactor but not molybdenum only.

Our experiments also indicated that molybdenum cofactor is available in the cytoplasmic extract of *E. coli* in a form which can be incorporated into subunit A and that no enzymatic activity is necessary to modify or insert cofactor into subunit A. Both the method of incubation (dialysis) with the extract and the lack of activity and antibody-precipitable enzyme in the extract preclude mere contamination of subunit A with the holoenzyme.

Third, only reconstituted subunit A had the spectral properties associated with non-cytochrome-containing enzyme (Fig. 2). These spectral properties were due to the nonheme iron content of reconstituted subunit A preparations. No nonheme iron was found associated with subunit B.

Subunits A and B of nitrate reductase are synthesized in the cytoplasm before insertion into the membrane (11, 23). Subunit B has been shown to be posttranslationally modified during this process of translocation. One of these changes is now known to be the covalent addition of fatty acid (unpublished data). The precursor-to-product change results in a decrease in the electrophoretic mobility of subunit B on SDS-polyacrylamide gels (24). A similar change in the electrophoretic mobility of subunit B also occurs during its release from the membrane by heat treatment (20, 21) as well as during purification (3). Depending upon the purification conditions, various amounts of modified nitrate reductase can be separated on a Bio-Gel column from complete nitrate reductase. Complete enzyme contains subunits A, B, and C. Modified enzyme contains subunits A and B' (greater mobility on SDS gels) but no subunit C. We have shown that this modification is caused by a membrane-bound enzyme which is removed during purification (Chaudhry and MacGregor, in press). It is not known whether similar changes also occur in subunit A, since subunit A from modified enzyme shows no change in mobility on SDS gels. We attempted to identify any changes in subunit A by comparing patterns of proteolytic digests of isolated subunit A from

unmodified, purified enzyme with those of subunit A from the cytoplasmic (precursor) and modified forms of the enzyme which contained modified subunit B. Proteolytic gel maps of precursor and modified forms of subunit A were identical to each other but were slightly different from the proteolytic gel map of subunit A isolated from unmodified enzyme (Fig. 4). From amino acid analyses of unmodified subunit A and precursor subunit A it was difficult to conclude whether there was any amino acid difference between these two polypeptides. Our results so far are consistent with the in vitro modification of subunit A (during purification) being the loss of a small covalently bound molecule and the in vivo change (cytoplasmic precursor form to membrane-bound form) being its addition. Similar results have been found with subunit B (Chaudhry and MacGregor, in press), for which proteolytic gel maps from modified and precursor forms of subunit B were similar to each other yet different from the proteolytic gel map of membrane-bound subunit B.

Recent studies in our laboratory show that fatty acids are covalently attached to both subunits A and B upon their insertion into the membrane (Richard Smith, unpublished data). This suggests that the events occurring during the modification of subunit A are probably similar to those occurring during the reversible conversion of B and B' (Chaudhry and MacGregor, in press), i.e., the addition of fatty acids and perhaps other molecules in vivo and their removal during purification by the modifying enzyme.

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