Vol. 105, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1982 Pages 450-456

EVIDENCE FOR THE PRESENCE OF A SMALL SUBUNIT AS THE PRINCIPAL COMPONENT OF THE NITRATE REDUCTASE OF ESCHERICHIA COLI K 12

P. FORGET[+] and M. DUBOURDIEU[++]

+ Laboratoire de Chimie Bactérienne, C.N.R.S., B.P. 71, 13277 MARSEILLE CEDEX 9 (France)

++ Universidad de Los Andes, Facultad de Ciencias, Grupo de Biologia Experimental, MERIDA (Venezuela)

Received December 7, 1981

An alkali and acid treatment of purified nitrate reductase from Escherichia coli yields a low molecular weight subunit. This subunit was characterized by its amino acid composition and its molecular weight (15000 dalton). The yield of the subunit production from native nitrate reductase reached 70 %. Important similarities were observed in the tryptic peptides maps of nitrate reductase and of the subunit. Several observations argue in favor of a repetitive structure in the nitrate reductase protein.

INTRODUCTION

Bacterial nitrate reductases are membrane-bounded respiratory enzymes which catalyze the reduction of nitrate to nitrite. Nitrate reductase of Escherichia coli has been solubilized and characterized by several authors (1, 2, 3, 4, 5). Enzyme solubilized by acetone treatment (1) has a molecular weight of 320 000 dalton and contains 2 atoms of molybdenum and 20 atoms of iron per molecule. Its absorption spectrum, with a plateau at 410 nm, resembles that of non-heme iron-sulfur proteins. The enzyme has been extensively studied with regard to its function and regulation (6, 7, 8) while its structural features are still uncertain. The first purification by organic solvent (1) did not indicate any subunit structure whereas all the following reports suggest several subunits. Most of them describe two types of subunits : a heavy one (about 150 000) and a lighter one (50 to 58 000) (2, 3). Polypeptides of different molecular weights (10 000, 45 000, 67 000) are also reported (3). Another subunit (22 000) seems to correspond to the apoprotein of cytochrome b (2).

At least three factors could be responsible for these disagreements about subunit composition of nitrate reductase : i) the variety of solubilization techniques used ii) the possible existence of a proteolytic action during the solubilization iii)some peculiar structural features. It is important to recall that this membrane protein contains carbohydrates (9), which makes sodium dodecyl sulfate polyacrylamide gel electrophoresis unreliable (10).

0006-291X/82/060450-07$01.00/0

In this work we describe a dissociating treatment of nitrate reductase which produces a low molecular weight polypeptide.

MATERIALS AND METHODS

Nitrate reductase purification was performed as published previously (1). The nitrate reductase used in this work was homogenous in polyacrylamide gel electrophoresis.

The pure native enzyme (1 mg/ml) was dissociated by the following process : First the protein was dissolved in 0.5 N NaOH. It was allowed to stand 5 min. at room temperature and then neutralized before dialyzing against a large volume of water, overnight at 4° C. The second step was a precipitation by HCl to a final concentration of 0.3N followed by a centrifugation. The colorless pellet was dissolved in 4 M Guanidine-HCl solution and then dialyzed against 1 % HCOOH/1 % SDS solution (protein concentration 5 mg/ml). At this step, the protein was filtrated on a Sephadex G-50 column equilibrated with 1 % HCOOH/1 % SDS. The resulting protein is called dissociated nitrate-reductase.

Electrophoresis in polyacrylamide gel (7 %) were performed according to Davis (11). In the presence of sodium dodecyl sulfate (SDS) a slight modification of the method of Weber and Osborn (12), (pH = 8.8, SDS = 1 %) was used. For molecular weight determination (12) protein standards were : cytochrome C553 (9000 M.W.) from Desulfovibrio vulgaris (13), Horse Cytochrome C (12 500) ; Deoxyribonuclease (13 500), Lysozyme (14 400) and pepsin (28 000). Before SDS electrophoresis proteins were incubated overnight in the electrophoresis 1 % SDS buffer and heated at 100° C for one minute just before the run.

Amino acid compositions were determined in an automatic amino acid analyser after hydrolysis under vacuum in 6 N HCl at 110° C for 15 hours. Cysteine and Cystine were estimated as cysteic acid after performic oxidation (14).

Tryptic peptides maps were obtained as follows. The dialyzed protein acid was submitted to three successive digestions with TPCK treated trypsin. Digestions were conducted for 10 to 15 hours in 1 % ammonium bicarbonate pH 8.5 and 37° C with an enzyme to substrate ratio of 1/100 (W/W). After each digestion the sample was heated at 100° C during 2 to 3 minutes. The resultant lyophilized material was then dissolved in 1 % pyridine and applied to a Whatman N° 1 paper for high voltage electrophoresis (3000 V at pH 6.5 for 35' and then at pH 1.9 perpendicularly to the first migration (15).

Proteins were determined with the Folin reagent by the method of Sutherland et al. (16).

RESULTS

The dissociation of nitrate reductase by the technique described in materials and methods consists of two steps. The alkali treatment changed the brown color of the protein solution to yellow but did not bleach it. The enzyme lost its color and its 410 nm absorption only after precipitation by 0.3 N HCl. It has to be noted that the same agents used in a different order might allow the precipitation of the protein with the concomitant impossibility of solubilizing it again, even in presence of urea or guanidium-HCl. The ultraviolet absorption spectrum, with a maximum near 278 nm was essentially unmodified by this treatment.

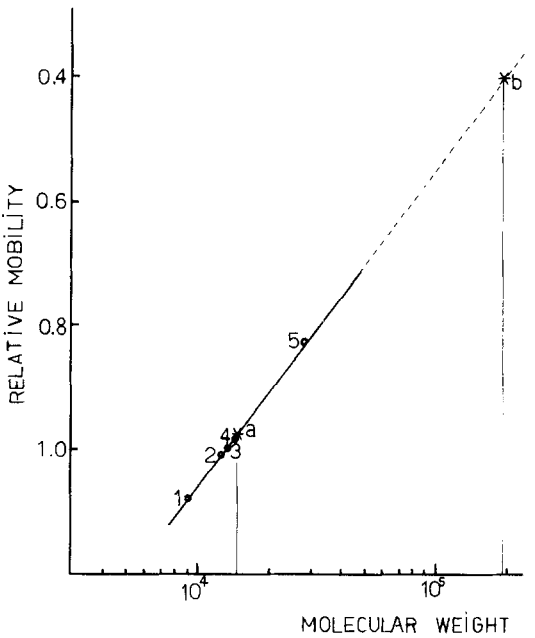

Figure 1. Molecular weight determination of the subunit S-15 by sodium dodecyl sulfate polyacrylamide gel electrophoresis.
Standards were cytochrome C_{553} (1), horse cytochrome (2), deoxyribonuclease (3), lysozyme (4) and pepsin (5).
a) Represents the subunit (14 800 dalton in this determination)
b) Represents the high molecular weight band (about 200 000). Mobility was measured with respect to bromophenol blue.

On Sephadex G-50 column the dissociated nitrate reductase was separated into two protein bands, with respective recoveries of 30 % for the first peak and about 50 % for the second smaller molecular weight peak. The recovery was calculated from the quantity of untreated nitrate reductase used in the denaturation. If the first eluting protein was submitted to a new denaturing treatment and newly chromatographed, more material of small molecular weight was obtained. At the end of the second denaturing treatment, 60 to 70 % of initial protein was recovered as this small molecular weight subunit which we will give the name subunit S-15.

The production of this subunit S-15 was also observed by electrophoresis in SDS polyacrylamide gels. While the untreated nitrate reductase gave at least five bands of unequal staining intensity (17), only two bands were observed with the dissociated protein : the subunit S-15 at the front and the other, minor component corresponding to a much higher molecular weight. The molecular weight of subunit S-15 was found to be 15 000 $\pm$ 1000 from several experiments (Fig. 1). The molecular weight of the other polypeptide was estimated between 150 000 and 200 000, with some variation from one assay to another.

The amino acid composition of subunit S-15 (Table 1) was consistent with a molecular weight of 14 000, since for such a molecular weight, whole

TABLE 1

AMINO ACID COMPOSITIONS OF NITRATE REDUCTASE AND OF THE FRACTIONS OBTAINED BY THE DISSOCIATING TREATMENT.

amino acid	Untreated NR		Dissociated NR		Subunit S-15		first fraction	
Lys	7.8	8	8.0	8	7.6	8	7.3	7
His	3.7	4	4.0	4	3.8	4	3.5	4
Arg	7.3	7	7.6	8	5.6	6	7.8	8
Cys	2.1	2	-	(2)	-	(2)		(2)
Asp	16.0	16	15.6	16	15.0	15	16.0	16
Thr	7.3	8	7.0	8	6.8	7	7.5	8
Ser	7.3	8	7.4	8	7.6	8	6.8	7
Glu	14.7	15	13.0	13	16.3	16	13.3	13
Pro	8.2	8	7.9	8	7.3	7	6.4	6
Gly	12.9	13	13.0	13	13.0	13	10.5	11
Ala	11.0	11	11.1	11	11.0	11	11.0	11
Val	7.3	7	6.7	7	6.0	6	6.4	6
Met	3.7	4	4.0	4	2.15	2	3.7	4
Ile	6.0	6	6.2	6	5.17	5	6.0	6
Leu	11.0	11	11.1	11	9.0	9	11.0	11
Tyr	5.5	6	5.8	6	3.8	4	5.0	5
Phe	3.7	4	3.1	3	3.2	3	3.2	3
Total		138		136		126		129
Corresponding MW		15 000		15 000		14 000		14 000

Amino acid compositions were calculated on the base of a molecular weight of 14 to 15 kilodalton. Stable aminoacids (Asp, Glu, Gly, Ala, Leu or Val) were used as references. The amino acid combination which gave the biggest proportion of whole numbers for stable amino acids was choosen if the corresponding composition fell between 125 and 145 residues. Tryptophane residues were not determined.

numbers were obtained for several of the stable amino acids (Asp, Gly, Ala, Val, Leu). Table 1 shows also that on the basis of 14 to 15 000 dalton molecular weight, the amino acid composition of subunit S-15 was very similar to the untreated and dissociated nitrate reductases, and also to the 150 000 dalton fraction. The most significant difference was found in glutamate content.

Untreated and dissociated nitrate reductase presented only one type of N- terminal residue, methionine, indicating that the dissociating process did not modify the primary structure. This result was not quantified.

The tryptic peptide map of nitrate reductase (0.8 mg of protein) is presented in Figure 2. Untreated and dissociated enzymes gave the same fingerprint. Some insoluble material is present at the origin of migration. 17

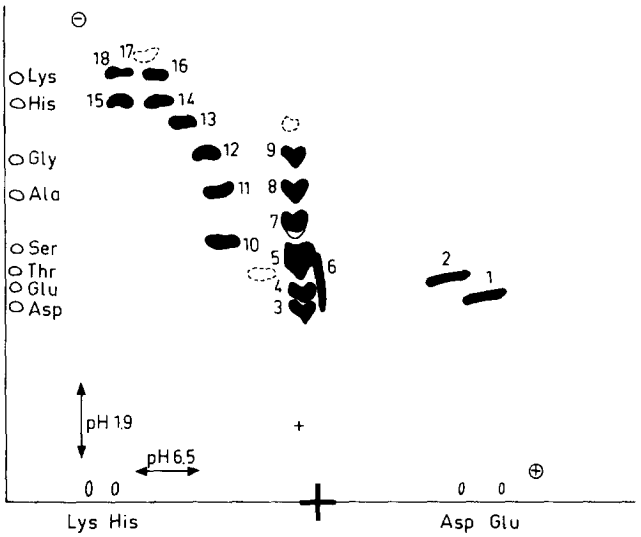

Figure 2. Fingerprint of nitrate reductase after tryptic digestion. 0.8 mg (about 5 nmoles on a 160 000 dalton basis or 50 nmoles if a 15 000 dalton basis is considered) was applied on a Whatman N° 1 paper (big cross). The black spots represent major peptides (at least 20 nmoles). Quantification was performed by comparing ninhydrin colour intensity of peptides with known amino acid standards. The little cross indicates the neutral position at pH 6.5 and 1.9. Some streaking was present at the application point.

major spots are shown, the intensity of which corresponds to 20 - 25 nmoles of peptide. This result may be surprising since some 170 peptides would be expected on the basis of a minimum molecular weight of 160 000. In such a case the concentration of each peptide would have been too small (2 to 3 nmoles) to be easily detected on the paper by ninhydrin. However the result agrees well with the 14 - 15 residues of lysine and arginine contained in 15 000 dalton subunits S-15 of nitrate reductase. The fingerprint of subunit S-15 presented essentially the same peptides as untreated nitrate reductase except for the peptides 10 and 16 which appeared as fain spots. Their size was estimated from their migration (18), assuming that these tryptic peptides had a double positive charge at pH 1.9, and yielded a total of some 100 residues. This result takes into account the possibility that some of these spots, might be free amino acids. The material precipitated on the paper near the origin could account for the remaining amino acids.

DISCUSSION

The dissociating procedure used in this study induced the production of a low molecular weight subunit S-15. Its molecular weight of 14 000 to 15 000 dalton was found to be consistent with its amino acid composition. The very surprising aspect of this study was that subunit S-15 appeared as a major component of nitrate reductase. The yield of production gave 60 per cent of

recovery of nitrate reductase in subunit S-15 form after one denaturing cycle, and more subunit S-15 was obtained upon submitting the 150 000 dalton polypeptide to a new denaturing treatment. The presence of a subunit S-15 which represents 60 to 70 per cent of nitrate reductase with 15 000 dalton molecular weight supposes a repetitive structure for the whole enzyme of E. coli.

Such a conclusion is strongly suggested by the following results : I) the above mentioned yield of production of subunit S-15 ; II) that the amino acid composition of subunit S-15 and untreated nitrate reductase were found to be very similar ; III) that the tryptic peptide maps of nitrate reductase and subunit S-15 present a strong similitude and the number of peptides obtained with nitrate reductase are in agreement with the number of basic residues contained in subunit S-15 ; IV) that a repetitive structure is strongly favoured by the fact that the ninhydrin coloured peptides would not have been detectable in the case of a 160 000 minimum molecular weight protein ; V) methionine was the only residue detected at the N-terminal for both subunit S-15 and untreated nitrate reductase.

We are conscious that each of these arguments may be criticized. For example the yield was determined by the Folin's method which may be considered unreliable for pure protein quantitation. Also the similarities in amino acid compositions and N-terminal residue cannot be considered as proofs. On the other hand some misinterpretation of the fingerprints cannot be discounted, since analysis of these peptides was not performed. However, we feel that these arguments taken together, particularly the fact that 5 nmoles of nitrate reductase can yield 20 - 25 nmoles of each trypsic peptide, indicate strongly a repetitive pattern in the structure of this protein.

The transient formation of a 150 - 200 000 dalton molecule during the denaturation suggests that the nitrate reductase molecule (320.000 MW , $2M_o(1)$) is formed of two subunits of 150 000. Such a molecule would then be a ten fold repetition of a 15 000 dalton subunit to which would be bound a molybdenum containing cofactor. Several association mechanisms can be considered to explain such a structure. Firstly it is obvious that the subunits S-15 are not linked solely by non covalent bonds because in such a case it would be difficult to explain why SDS cannot desaggregate untreated nitrate reductase into subunit S-15. Likewise the subunits S-15 are not covalently linked through disulfide bridges ; β-mercaptoethanol does not modify the number of subunits in any of the denaturing technique used. But it is possible to retain two types of bond. The subunits S-15 could be linked through iron-sulfur clusters. Nitrate reductase contains one atom of iron per subunit S-15 (1) and the acid denaturing step used to prepare the subunit S-15 corresponds to the classical apoprotein preparation of non-heme iron proteins. The second possibility corresponds to a bond through sugar molecules. Nitrate reductase has been described as a glycopro-

tein (9). The denaturing NaOH step can hydrolyse some type of carbohydrate - protein bond such as ester linkages (19).

The claim of a repetitive pattern in the structure of nitrate reductase is quite radical since it implies that some sort of covalent linkage, different from disulfide bridge, is binding the subunits S-15 together.

ACKNOWLEDGEMENTS :

We are indebted to Dr. M. Scandellari and R. Bourrelli for growing the bacteria. We gratefully acknowledge the skillful technical assistance of R. Rimassa. We thank CNRS (France) and CONICIT (Venezuela) for financial assistance.

REFERENCES

1. Forget, P. (1974) Eur. J. Biochem. 42, 325-332.
2. Mac Gregor, C.H., Schnaitman, C.A., Normansell D.E. and Hodgins, M.G. (1974) J. Biol. Chem. 249, 5321-5327.
3. Lund, K. and DeMoss, J.A. (1976) J. Biol. Chem. 251, 2207-2216.
4. Clegg, R.A. (1975) Biochem. J. 153, 533, 541.
5. Enoch, H.G. and Lester, R.L. (1975) J. Biol. Chem. 250, 6693-6705.
6. Pichinoty, F. (1973) Bull. Inst. Pasteur 71, 317-395.
7. Payne, W.J. (1973) Bacteriol. Reviews 37, 409-452.
8. Ruiz-Herrera, J. and DeMoss, J.A. (1969) J. Bacteriol. 99, 720-729.
9. Forget, P. (1977) F.E.B.S. Letters 77, 182-186.
10. Wong, M.M., Robertson, N.P. and Horwitz, J. (1978) Biochem. Biophys. Res. Commun. 84, 158-165.
11. Davis, B.J. (1964) Ann. NY Acad. Sci. 121, 404-410.
12. Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.
13. Bruschi, M. and Le Gall, J. (1970) Biochem. Biophys. Res. Commun. 38, 607-616.
14. Moore, S. (1963) J. Biol. Chem. 238, 235-239.
15. Ambler, R.P. (1963) Biochem. J. 89, 349-378.
16. Sutherland, E.W., Cori, C.F., Haynes, R. and Olsen, M.S. (1949) J. Biol. Chem. 180, 825.
17. Grassi, C. Andrade, E., Puig, J. (1979) Biochem. Biophys. Res. Commun. 90, 86-91.
18. Brown, J.R. (1968) Southwest Regional ACS Meeting Abstract, Austin, Texas.
19. Pigman, W. in Pigman (1957) The carbohydrates p. 406, Academic Press Ed. New York.