EVIDENCE FOR THE PRESENCE OF CARBOHYDRATE UNITS IN THE NITRATE REDUCTASE A OF *ESCHERICHIA COLI* K12

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1. Introduction

The nitrate reductase of *Escherichia coli* is a membrane-bound respiratory enzyme classified as a nitrate reductase A [1]. Simple washing of intact cells or osmotic shock on cytoplasmic membrane does not release any of the enzyme. It has been possible to solubilize it by means of a detergent as Triton X-100 [2], the action of a protease [3] or the combined effect of acetone and sodium deoxycholate [4]. It appears, from the different purification procedures recently published, that nitrate reductase is a protein tightly bound to the cytoplasmic membrane. According to the classification suggested by Singer [5], nitrate reductase is an integral protein rather than a peripheral one such as the cytochrome *c* of mitochondrial membrane which is detached by simple washing with a 3 M KCl solution [6].

The different treatments used to solubilize the nitrate reductase, show that this enzyme is strongly associated with the lipid layer of the membrane. So, we can expect to find in this protein some characteristics related to the amphipatic structure of the integral proteins [5]. For this reason we have looked for some common non-protein components. Preliminary results suggest that the nitrate reductase has a much more complex structure than we had initially considered.

2. Materials and methods

2.1. *Cultures and enzyme preparation*

Escherichia coli strain K12 PA602 is grown in a 200 l fermenter. The growth conditions, crude extract preparation and nitrate reductase purification have been described in a previous publication [4]. The homogeneity of enzyme preparations is checked by polyacrylamide gel electrophoresis.

2.2. *Acid hydrolysis of sugars and purification on ion-exchange resin*

After 24 h dialysis against distilled water, the protein is hydrolysed with HCl in a sealed tube in vacuo. Hydrolysis conditions are: 1.5 N HCl, 1 h at 100°C. The solution is then diluted with distilled water up to 0.3 N HCl and chromatographed on a column of Dowex 50 X 8 (200–400 mesh) H^+-form and then on a second column of Duolit A 102 D, acetate form. Both columns (100 ml each) are washed first with 500 ml distilled water, then with 500 ml 0.3 N HCl solution. Neutral sugars are eluted by the first washing while amino sugars come out with 0.3 N HCl. The two fractions are concentrated before to be used for different analysis.

2.3. *Paper chromatography of neutral sugars*

Paper chromatography of neutral sugars is carried out, using Whatman paper No. 1, in a descending system solvent composed of pyridine/ethyl acetate/water (v/v/v; 2/2/1). Sugars are revealed on the chromatogram by aniline oxalate reagent at 110°C. Hexoses give a brown spot and pentose a pink spot [8].

2.4. *Identification and determination of hexosamines with an automated amino acid analyzer*

Hexosamines are eluted from resin column in the course of amino acid analysis and emerge toward the end of the elution of neutral amino acids. The column

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of 42 cm is loaded with cation exchange resin Durrum D C-6A. Temperature is 51°C at the beginning and shifted up to 78°C after 42 min. Three different citrate buffers are used successively: pH 3.18 (14 min), pH 4.22 (29 min) and pH 6.55 (33 min). In these conditions glucosamine emerges at 74 min galactosamine at 77 min. Phenylalanine emerges just before the two osamines at 72 min.

2.5. *Polyacrylamide gel electrophoresis*

Polyacrylamide gel electrophoresis is carried out at pH 8.8 according to the method of Davis [7]. Gels are stained with Aniline Blue Black for proteins and with periodic acid Fuchsin reagent for glycoproteins [9].

2.6. *Analytical techniques*

Proteins are evaluated by Folin phenol reagent [10] using bovin serum albumin as standard. Neutral sugars, amino sugars and sialic acid are determined respectively by Dische and Shettles method [11], the Elson Morgan procedure [8] and by thiobarbituric acid reaction [12].

3. Results and discussion

3.1. *Polyacrylamide gel electrophoresis and detection of carbohydrates by periodic acid Fuchsin reagent*

In order to determine the qualitative presence of carbohydrate, electrophoresis is carried out simultaneously on two polyacrylamide gels. On the first gel, the homogeneity of the nitrate reductase preparation is tested; only one protein band is stained by Aniline Blue Black (fig.1, Tube 1). Contaminating protein, if any, does not exceed 2 or 3 percent. The second gel is stained for glycoprotein by a method using the periodic acid Fuchsin reagent, which is specific for polyalcohol groups. Under these conditions, the development of a red coloration located at the same position as nitrate reductase in the first gel (Tube 2, fig.1), demonstrates the presence of sugars associated to the protein. During electrophoresis, both components migrate together and thus nitrate reductase appears as a protein containing a tightly bound glycan moiety.

Fig.1. Polyacrylamide gel electrophoresis. (I) Coloration by Aniline Blue Black. (II) Coloration by periodic acid Fuchsin reagent. Gel concentration: 7%. Buffer HCl–Tris/glycine, pH 8.8. Anode at bottom.

3.2. *Estimation of neutral sugars, amino sugars and sialic acid*

We have looked for neutral sugars, amino sugars and sialic acid, in the native protein by specific colorimetric techniques. It is well known, that in a strong acid medium, the sugars of a glycoprotein are hydrolysed and decomposed at 100°C to a furfuraldehyde compound which reacts with cysteine to give characteristic chromogen with a maximum absorption

at 396 nm for pentose, 400 nm for methylpentose and 410 nm for hexose. The proteins do not interfere during this reaction and it is thus possible to characterize and estimate the neutral sugars of glycoprotein. Nitrate reductase gives a positive reaction and the spectrum of the chromogen has an absorption maximum at 410 nm, characteristic of a hexose. Using glucose as a standard, the estimated hexose content represents 3–5% by weight protein (table 1).

The chemical estimation of hexosamine has been performed by the Elson Morgan procedure [8] after acid hydrolysis. The quantity of amino sugars contained in the nitrate reductase represents 4–5% protein weight with glucosamine as standard. Furthermore, when the reaction occurs without acid hydrolysis of the protein prior to the estimation by Ehrlich reagent, no amino sugar is detectable. As this reagent reacts only with free amino sugars, it appears that a glycosidic-bond links the amino sugar with the nitrate reductase peptidic chain.

In order to check if the presence of protein can modify the sugar estimation, the protein was previously discarded by ion-exchange chromatography before the determination of sugars. The yield of neutral sugars is lowered by this purification step which may have produced some losses. But it is also possible that the protein interferes and gives an overestimation of neutral sugars. With the osamines, in both cases, the results are the same and it is likely that the osamines represent about 5% by weight of the protein.

Thiobarbituric acid is used to detect sialic acid [12]. As in the case of osamines, the preparation is first submitted to a mild acid hydrolysis as generally used for such purpose. No sialic acid is detectable by this method (table 1). This negative result also eliminates the presence of 2-deoxyribose which reacts under the conditions used to detect sialic acid.

3.3. *Identification of neutral and amino sugars*

We have already put in evidence the presence of hexoses in the nitrate reductase by the Dische and Shettles reaction [11]. Two different analytical methods have been used to identify these compounds.

The hydrolyzed protein is chromatographed on an ion exchange resin and the deionized fraction used for paper chromatography analysis. Aniline oxalate reagent revealed the presence of two sugars. Glucose is the most important; the other one gives the same pink spot as a pentose.

Examination of carbohydrates by gas–liquid chromatographs (GLC) reveals the presence of glucose and glycerol. It is also possible to see on the GLC-chromatogram the presence of traces of galactose but no pentose is visible (fig.2, Chromatogram 1).

So glucose presence in our nitrate reductase is well established by two different analytical techniques.

Amino sugars are also identified by two different methods. Examination of the purified amino sugar fraction has been carried out on an automated amino acid analyzer. Only one amino sugar is characterized. It emerges from the column at the same time as glucosamine. If glucosamine is added as an internal standard both compounds emerge as a unique peak. Moreover the quantity of amino sugar determined by the Elson Morgan procedure on the analyzed sample is quantitatively recovered in the

Table 1
Determination of carbohydrate compounds (mg/100 mg protein)

	Neutral sugar[a]		Amino sugar[b]		Sialic acid
	A	B	C	B	D
Essay 1	3.5	2.5	3.0	4.7	ND
Essay 2	5.5	1.8	4.6	4.5	ND

[a] Glucose as standard
[b] Glucosamine as standard

Analyses are carried out: A, on the whole protein; B, after hydrolysis and purification of sugar fraction (see Materials and methods); C, after hydrolysis (1.5 N HCl, 1 h 30 min, 100°C); D, after hydrolysis (0.01 N H_2SO_4, 1 h, 100°C.

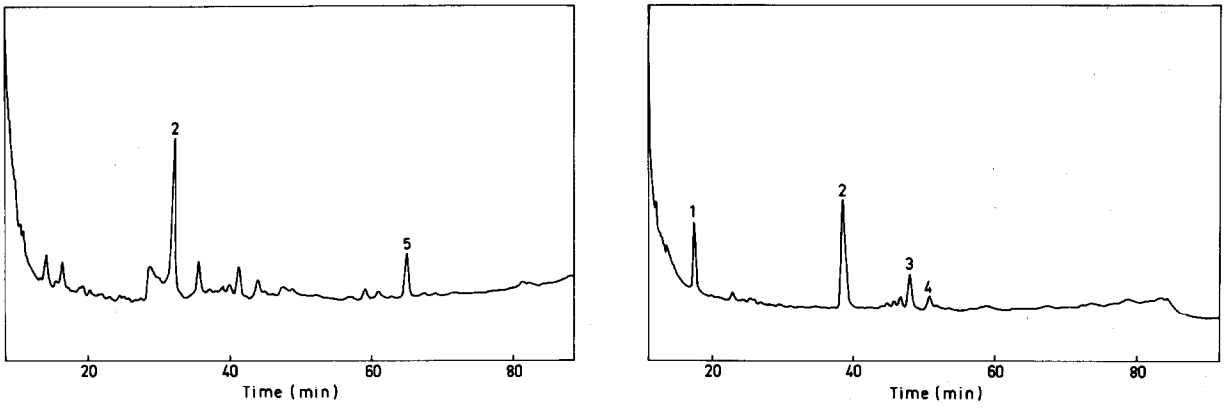

Fig.2. Gas-liquid chromatography (GLC). (a) Chromatogram I. Analysis of neutral sugar fraction, purified on ion-exchange resin (see Materials and methods) then submitted to methanolysis (MeOH/HCl 0.5 N, 80°C, 24 h) and trifluoroacetylated (methylene chloride and trifluoroacetic anhydride v/v, 150°C, 5 min) before analysis. The GLC conditions are as follows: liquid phase, silicone OV 210 (5%); temperature gradient 110–220°C; programming rate 1°C/min; carrier-gas nitrogen at a flow-rate of 50 ml/min; internal standard mesoinositol. (b) Chromatogram II. Analysis of amino sugar fraction. Sample is peracetylated (pyridine/acetic anhydride (v/v)) before analysis. Methanolysis and GLC conditions as described above. Peaks are identified as glycerol 1; mesoinositol 2; glucose 3, 4; glucosamine 5.

glucosamine peak of the amino acid analysis. So we can expect that glucosamine is the only amino sugar of the nitrate reductase.

Gas–liquid chromatography analysis of our sample shows also the presence of glucosamine but no trace of other amino sugars (fig.2, Chromatogram II). The other peaks on chromatogram II correspond to amino acids which are eluted from the Dowex column at the same time as glucosamine.

4. Conclusion

We have obtained some evidence for the presence of carbohydrate units in homogeneous nitrate reductase preparations. On polyacrylamide gel electrophoresis stained by periodic acid Schiff reagent, we have characterized the presence of a glycan moiety in the protein. Different analytical methods allowed us to identify glucose and glucosamine but no mannose which is often present in glycoproteins.

Recently in the membrane bound ATPase of *Micrococcus lysodeikticus* Andreu et al. [13] found two sugars: glucosamine and a hexose. On the other hand, it is suggested by Guidotti [14] that membrane-transport processes would be catalyzed by transmembrane oligomeric glycoproteins. Therefore, it is possible that many membrane bound proteins are glycoproteins. The role of sugars is not known, however they might play a role of protection against proteases. We have observed that hydrolysis of nitrate reductase by trypsin is anomalously low without a preliminary denaturation of the protein by heating.

The glycoproteic nature of the nitrate reductase is a new feature which has to be taken into account for a structural study of the enzyme. For instance, it is well known that some glycoproteins interact with sodium dodecyl sulfate (SDS) differently than non-glycosylated proteins [15]. They bind, on a weight basis, less anionic detergent and it could be expected a great difference in the molecular shape between glycoproteins and non-glycosylated proteins. Therefore, to estimate the molecular weight of a glycoprotein SDS–polyacrylamide gel electrophoresis or high speed equilibrium sedimentation should be used with caution [16].

After this preliminary work, to establish the presence of carbohydrate units in the nitrate reductase, we expect to characterize the type of carbohydrate–peptide linkage involved in the protein.

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