# STUDIES ON ATP-DEPENDENT DEOXYRIBONUCLEASE OF *Haemophilus Influenzae* : INVOLVEMENT OF THE ENZYME IN THE TRANSFORMATION PROCESS.

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Summary : An ATP-dependent deoxyribonuclease deficient mutant of *Haemophilus influenzae* has been isolated on the basis of sensitivity to methyl methane sulfonate (mms). Furthermore, the involvement of the ATP-dependent deoxyribonuclease in the process of transformation in *H. influenzae* has been demonstrated. Among the 75 mms-sensitive mutants, two mutants lacking ATP-dependent deoxyribonuclease were isolated. One mutant Rd/mms-s 21 has no measurable ATP-dependent deoxyribonuclease activity, is almost transformation deficient and has normal DNA uptake after being submitted to competence protocole. The second ATP-dependent deoxyribonuclease deficient mutant which has abnormal DNA uptake is still under investigation. Furthermore, the enzyme deficient mutant mms-s 21 transformed back to mms-resistance, recovers the ATP-dependent deoxyribonuclease as well as the transforming ability. The involvement of the ATP-dependent deoxyribonuclease in the process of transformation in *H. influenzae* is therefore positively established.

Recently several investigators reported the presence of an ATP-dependent deoxyribonuclease in organisms such as *Escherichia coli* (1, 2, 3, 4, 5, 6, 7), *Diplococcus pneumoniae* (8, 9), *Micrococcus luteus* (10, 11, 12), *Haemophilus influenzae* (13, 14, 15) and *Mycobacterium smegmatis* (16). The involvement of the ATP-dependent deoxyribonuclease has been demonstrated in recombination (2, 4, 6, 7, 9) in DNA repair (10) and in transformation (17). In *H. influenzae* this enzyme has been purified (13) but its involvement in transformation has not yet been demonstrated. This prompted us to look for mutants of *H. influenzae* lacking this enzyme. Indeed, our mutant Rd/mms-s 21 which is ATP-dependent deoxyribonuclease deficient and transformation deficient, allowed us to draw a parallel between the lack of this enzyme and the strong reduction of transforming ability.

## MATERIALS AND METHODS :

Strains : strains used were wild type *H. influenzae* Rd, provided by Dr. R.M. Herriott, and mutants of this strains isolated in our laboratory :

Rd/mms-sensitive 21

Rd/mms-resistant $21_1$ - $21_2$ - $21_3$ - $21_4$, obtained by transformation of Rd/mms-s 21 with wild type DNA.

Media : standard growth medium was brain-heart infusion (35°/₀₀) diluted 1:1 with eugon broth (30°/₀₀) and supplemented with hemin and NAD (5 µg/ml). For solid medium 15 g °/₀₀ of bacto agar were added.

Isolation of mms-sensitive mutations : Cultures of strain Rd grown to about $1 \cdot 10^9$ cells in liquid growth medium were at first exposed to nitrosoguanidine (200 µg/ml of culture, for 30 min. at 37°C) and then allowed to growth overnight. The cultures were then plated on brain-heart infusion agar to yield about 200 colonies/plate and grown overnight at 37°C. The colonies were then replica-plated onto brain-heart infusion agar plates and on plates supplied with 0.1 M methyl methane sulfonate. Only colonies which failed to grow on the latter plates were picked as mms-sensitive mutants and tested furthermore.

Competence and transformation procedure : Competent cells were prepared according to the method of Cameron as described by Barnhart and Herriott (18). Homologous donor DNA carrying the novobiocin resistance marker (nov-r) was isolated according to the method of Notani, Frankel and Goodgal (19) and purified using the method of Greth and Chevallier (20). For transformation assays 0.5 ml of (nov-r) DNA (1 µg/ml) was added to 4 ml of liquid brain-heart infusion medium and to 0.5 ml of competent recipient bacteria. The suspension was incubated for 2 hours at 37°C in a water bath and plated onto agar plates supplemented with the required inhibitor : 0.1 M methyl methane sulfonate or 1 µg/ml novobiocin.

Preparation of $^3$H-labeled DNA : $^3$H-labeled DNA (9,000 CPM/µg) was extracted from a thy$^-$ derivative of *H. influenzae* grown in synthetic medium similar to the one described by Herriott (21) and supplemented with 0.4 µci/ml of $^3$H-methyl thymidine (specific activity 7.5 ci/mmole) from C.E.A. France, and with 5 µg/ml of cold thymidine. Cells were lysed by the method of Notani and Goodgal (19). Further purification of the DNA was performed by the method of Greth and Chevallier (20).

Preparation of enzyme extracts : 10 ml of a culture with an optical density of 0.40 - 0.50 at 650 nm in a Coleman spectrophotometer were centrifuged for 10 min. at 10,000 RPM in a refrigerated centrifuge type Sorvall. The pellet was washed twice in 0.01 M tris-HCl, 0.15 M NaCl pH 7.8 and resuspended in 2 ml of 0.01 M tris-HCl pH 7.8, 0.001 M 2-mercaptoethanol. The suspension was then treated for 3 hours in the presence of 50 µg of a freshly prepared solution of lysozyme. The resulting viscous suspension was then centrifuged for 30 minutes at 10,000 RPM and directly used. Samples can be stored at -74°C after addition of a final concentration of 20% of glycerol.

TABLE 1

Change in ATP-dependent deoxyribonuclease activity in wild type strain Rd and mutant derivatives.

| Strains | Enzymatic activity without ATP in cpm/ml | Enzymatic activity with ATP in cpm/ml | ATP-dependent activity in cpm/ml | Units/ml | Protein in mg/ml | Specific activity (units/ml / mg protein) |
|---|---|---|---|---|---|---|
| Wild type Rd | 540 | 15,180 | 14,640 | 1.63 | 2.5 | 0.65 |
| Wild type Rd | 1,550 | 11,750 | 10,200 | 1.34 | 2.8 | 0.48 |
| mms-sensitive strain 21 | 574 | 559 | < 0 | | 2.3 | |
| | 561 | 557 | < 0 | | 3.4 | |
| | 573 | 537 | < 0 | | 2.9 | |
| mms-resistant strains 21-1 | 1,020 | 10,460 | 9,440 | 1.10 | 2.4 | 0.46 |
| 21-2 | 870 | 13,800 | 12,930 | 1.45 | 2.6 | 0.56 |
| 21-3 | 1,160 | 15,660 | 14,500 | 1.61 | 1.8 | 0.89 |
| 21-4 | 820 | 16,090 | 15,270 | 1.70 | 2.6 | 0.66 |

For all these strains numerous other assays giving similar results were performed.

For the definition of enzyme unit, see results, first paragraph.

Enzyme assay : In the routine assay the reaction mixture (0.5 ml) contained 0.1 ml of enzyme extract, 60 µmoles of tris-HCl buffer pH 7.8, 2 µmoles of $Mg\ Cl_2$, 5 µmoles of 2-mercaptoethanol, 0.5 µmoles of ATP (sodium salt ; the solution was neutralized to pH 7 with Na OH N/10) and 0.5 µg of $^3H$-labeled DNA. Blanks without ATP were always run in parallel. The mixture was incubated in a water-bath at 37°C for 30 minutes. Under the experimental conditions the assay was linear with respect to amount of enzyme and time. The reaction was stopped by addition of 0.2 ml of calf thymus DNA (O.D. 40) and 0.2 ml of 12% perchloric acid. Samples were kept on ice for 10 minutes, then centrifuged for 20 minutes at 10,000 RPM and 0.5 ml of the supernatant was counted in 8 ml of aquasol (New England Nuclear - Boston - Mass.) with a Beckman liquid scintillation spectrometer.

For the determination of the specific activity, protein concentration was evaluated by the method of Lowry et al. (22) with bovine serum albumin as a standard.

DNA uptake and transformation : Incubation mixture contained 0.5 ml of highly competent cells, 0.3 ml of $^3H$ DNA (10 µg/ml) and 4 ml of saline (Na Cl 0.15 M pH 7.2). After 10 minutes of incubation at 37°C, bovine pancreas deoxyribonuclease was added to a final concentration of 10 µg/ml, and the incubation was continued for another 10 minutes. The mixture was then centrifuged at 10,000 RPM and washed twice with saline. The pellet was then dissolved in 0.5 ml of saline and was counted in 8 ml of aquasol with a Beckman liquid scintillation spectrometer. Before the addition of the deoxyribonuclease an aliquot was removed, diluted 10 times in brain-heart infusion medium, incubated at 37°C for 90 min. and plated onto agar plates supplied with novobiocin (1 µg/ml) for transformation assay. Blanks with non competent bacteria and approximately the same optical density were run in parallel.

RESULTS :

Change in ATP-dependent deoxyribonuclease activity in wild type strain Rd and in mms-sensitive and mms-resistant mutant strains : Table 1 shows 1) the presence of enzymatic activity in wild strain Rd, 2) the lack of enzymatic activity in the transformation deficient mutant strain mms-s 21, 3) and its complete recovery in strains obtained after transformation of strain mms-s 21 back to mms resistance. The assays were run in the presence or absence of ATP under the standard conditions previously described in materials and methods. One unit of enzyme is defined as the amount of activity that results in the degradation of 1 µg of DNA ($9 \cdot 10^3$ cpm of $^3H$ thymine) to acid-soluble fragments in 20 minutes at 37°C. Specific activity is defined as one unit per mg of protein.

TABLE 2

Change in transformation frequency.

| Strain | Transformation frequency (% of wild type) |
|---|---|
| Wild type Rd | 100 |
| mms-sensitive 21 | 0.78 - 0.89 - 0.89 |
| mms-resistant 21-1 | 108 - 130 - 96 - 103 |

The transformants were obtained by transforming recipient competent cells of wild type strain Rd and mutant strains mms-sensitive and mms-resistant with homologous (nov-r) DNA. The indicated results affect separate experiments with every time freshly prepared competent recipient cells.

Change in transformation frequency : The conditions for the transformation assays have been described in materials and methods. Competent recipient cells were treated with nov-r donor DNA and the novobiocin transformation frequency was determined. Revertants have not been detected in the standard conditions. It must be noted that the time of growth of strain Rd and of its mutant strains was almost the same (30 to 45 minutes for one cell division in standard liquid medium, at 37°C and under shaking).

Table 2 shows that the mms-sensitive strain 21 which is entirely free of ATP-dependent deoxyribonuclease activity exhibits a dramatic decrease of transformation frequency, as compared to the wild type parent.

On the other hand table 2 shows that the isolated mms-resistant strains recover simultaneously the enzymatic activity and the transformation ability.

DNA uptake and transformation : The incubation mixture were as described under materials and methods. Table 3 shows that there is no significant difference

TABLE 3

DNA uptake and transformation.

| | Competent wild type strain Rd | Non competent wild type strain Rd | Competent mutant strain mms-s 21 | Non competent mutant strain mms-s 21 |
|---|---|---|---|---|
| cpm irreversibly bound per $1 \cdot 10^8$ bacteria | 1,102 | 480 | 940 | 495 |
| viable colony-forming units/ml | $4.2 \cdot 10^8$ | $4.7 \cdot 10^8$ | $3.32 \cdot 10^8$ | $3.73 \cdot 10^8$ |
| novobiocin-resistant transformants /ml | $1.6 \cdot 10^5$ | $4 \cdot 10^1$ | $1.42 \cdot 10^3$ | $1 \cdot 10^1$ |

This table shows the similar uptake of DNA in both studied competent strains as well as the considerably lowered level of transformation frequency in the mutant strain.

in the level of uptake in wild strain Rd and in mutant strain mms-sensitive 21, but that the transformation frequency is considerably lowered in the mutant strain compared with the transformation frequency of wild type strain Rd. The DNA is well entering in both strains but he is poorly integrated in the mutant strain.

CONCLUSION : The isolation of mutant strain Rd-mms-s 21 transformation deficient, ATP-dependent deoxyribonuclease deficient, as well as tests on enzymatic activity and transformation assays, enabled us to assert that the ATP-dependent deoxyribonuclease is effectively required in normal transformation process in *H. influenzae*. Nevertheless a small residual transforming activity is remaining in the strain mms-s 21. It cannot yet be precised if this activity is due to a very low residual enzymatic activity or to a mecanism with low efficiency which can operate without this enzyme.

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