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# NADH and NADPH-Viologen Reductases From *Acinetobacter calcoaceticus* *

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Three pyridine nucleotide-dependent diaphorases have been isolated from *Acinetobacter calcoaceticus* cells and partially characterized. Two of them, with molecular weights of 165,000 and 57,000, utilize NADPH as electron donor whereas the third one (MW = 57,000) is specific for NADH. Oxidized viologen dyes, flavin nucleotides, dichlorophenol indophenol and ferricyanide can act with efficiency as acceptors in the reaction mediated by these diaphorases. The diaphorase activities have been characterized kinetically, and the effect of different inhibitors and cofactors has been also studied. The diaphorases seem to be subjected to metabolic control by oxidation and reduction.

The assimilatory nitrate reductase from bacteria and blue-green algae described until now can use reduced viologen dyes as electron donors for the reduction of nitrate to nitrite (4, 6, 12, 21). Although in blue-green algae ferredoxin reduced by electrons coming from the photosynthetic splitting of water is the physiological electron donor for the reduction of nitrate (12, 16), the question remains open whether this electron carrier is also functional in the process in photosynthetic and non-photosynthetic bacteria. Actually reduced ferredoxin can act as the immediate electron donor for the enzymatic reduction of nitrate in some photosynthetic and non-photosynthetic bacteria (3, 11, 19).

In previous works we have characterized the nitrate reductase of *Acinetobacter calcoaceticus* and have shown that viologens and *Acinetobacter* ferredoxin reduced either chemically or by extraneous ancillary systems (illuminated

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spinach chloroplasts, spinach NADP * reductase) can act as effective donors in enzymatic reduction of nitrate (20, 21).

In this communication, the isolation and partial characterization of three pyridine nucleotide-dependent diaphorases present in *A. calcoaceticus* cells are reported, and their possible role in the enzymatic reduction of nitrate is discussed.

## Materials and Methods

*Cells culture.* *Acinetobacter calcoaceticus* cells (strain 3 from Instituto de Biología Celular, Madrid, culture collection) were cultivated aerobically as earlier described (21).

*Preparation of cell-free extracts.* The bacterial cells were harvested at the end of the logarithmic phase of growth by centrifugation at 16,000 × *g* for 5 min, resuspended, after washing twice with distilled water, with 50 mM potassium phosphate buffer, pH 7.5, 10 μM in FAD, and disrupted in the cold with a sonifier Branson-12 (20 kHz/75 W) for 5 min. The broken cells were spun down at 39,000 × *g* for 15 min, and the resulting supernatant was used as starting material for enzyme purification. In these crude extracts nitrite reductase activity was undetectable.

*Enzyme purification.* The cell-free extract was brought up to 25 % ammonium sulfate saturation by stepwise addition of the salt. During ammonium sulfate precipitation, pH 7.5 was maintained by addition of $K_2HPO_4$ 1 M. The 25 % saturated suspension was centrifuged at 27,000 × *g* for 15 min, and the resulting supernatant was supplemented with $(NH_4)_2SO_4$ up to 50 % saturation. After centrifugation at 27,000 × *g* for 15 min, the sediment was dissolved with potassium phosphate buffer 20 mM, pH 7.5, 10 μM in FAD (Fraction I), and the supernatant was subjected to further precipitation with $(NH_4)_2SO_4$ up to 80 % saturation. After sedimentation and solubilization in the foregoing conditions, a second enzyme fraction was obtained (Fraction II). Both fractions were then passed separately through a Sephadex G-150 column (33 × 3 cm) equilibrated with potassium phosphate buffer 50 mM, pH 7.5, 10 μM in FAD, and 3 ml fractions were collected and assayed for enzyme activities. After this purification procedure the specific diaphorase activities were 4 times higher than in the crude extract and, in addition, showed a greater stability when stored for 4 weeks at −20° C. When FAD-free enzyme preparations were needed, an additional filtration through a Sephadex G-25 column (7 × 2.5 cm) equilibrated with potassium phosphate buffer 50 mM, pH 7.5, was performed.

*Enzyme assays.* Nitrate reductase activity was assayed as previously described (21). The standard assay for diaphorases was carried out spectrophotometrically at room temperature by following the absorbance changes of oxidized DPIP at 600 nm. The reaction mixture included, in a final volume of 2 ml, potassium phosphate buffer, pH 7.5, 200 μmoles; DPIP, 0.2 μmoles; NAD(P)H, 0.6 μmoles, and sufficient amount of enzyme. The activity in the presence of menadione and flavins was measured by following the oxidation of NAD(P)H at 340 nm. When other acceptors were used, the reaction was also carried out spectrophotochemi-

* Abbreviations: $NAD(P)^+$, NAD(P)H = Nicotinamide adenine dinucleotide (phosphate) and its reduced form; FN = Flavin nucleotide; FMN = Flavin mononucleotide; FAD = Flavin adenine dinucleotide; *p*-HMB = *p*-hydroxymercuribenzoate; DPIP = 2,6-dichlorophenolindophenol; Tris = Tris (hydroxymethyl) aminomethane; BV = Benzyl viologen; MV = Methyl viologen; MW = Molecular weight; $NR_{ase}$ = Nitrate reductase.

cally by observing the absorbance changes at the following wavelengths: ferricyanide, 420 nm; cytochrome *c*, 550 nm; BV, 565 nm, and MV, 602 nm. The assay with the viologens was performed in Thunberg tubes repeatedly evacuated and filled with $O_2$-free $N_2$. The molar extinction coefficients ($E^{1\,M}_{1\,cm}$) used in calculations were: 21,000 for DPIP; 6,300 for NAD(P)H; 28,000 for cytochrome *c*; 8,300 for BV, and 9,700 for MV.

Enzyme units are defined as $\mu$moles of NAD(P)H oxidized or substrate reduced per min.

*Analytical methods.* Growth was estimated turbidimetrically by following the absorbance changes of the cultures at 660 nm. Protein was determined with the Folin-phenol reagents using bovine serum albumin as standard (10). Nitrite was measured by diazotation as reported elsewhere (21). The molecular weigth was calculated from gel filtration data using a Sephadex G-150 column (66.5 × 5 cm) according to ANDREWS (1). The standard proteins used to calibrate the column; were: human-$\gamma$-globulin (MW = 160,000); bovine serum albumin (MW = 67,000); ovalbumin (MW = 45,000); $\alpha$-chymotrypsinogen A (MW = 25,000), and horse cytochrome *c* (MW = 12,500).

## Results and Discussion

In crude extracts of *A. calcoaceticus* cells there are, in addition to nitrate reductase, three diaphorases physically separable by gel filtration (fig. 1 A). Two of them are NADPH-dependent and the third one utilizes only NADH as electron donor. These three activities can be separated by $(NH_4)_2SO_4$ fractionation followed by gel filtration as can be seen in figure 1 B and 1 C.

Diaphorase activities were proportional to the amount of enzyme used in the assay. When the reaction was carried out in the presence of boiled extracts (100° C, 5 min), no activity was detected. In addition, the reaction showed in all the cases a linear kinetics with the time during at least 30 min (results not shown).

According to their elution order from the Sephadex column, the diaphorase activities can be named NADPH-diaphorase I, NADPH-diaphorase II and NADH-diaphorase.

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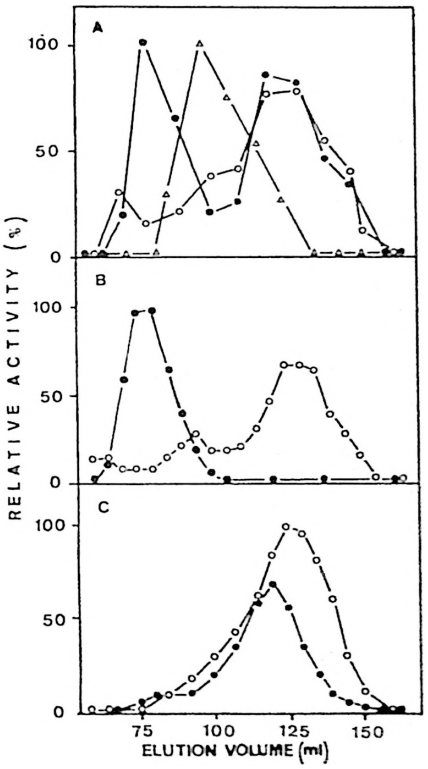



Fig. 1. *Elution pattern from gel filtration of nitrate reductase and NAD(P)H-viologen diaphorases from* Acinetobacter calcoaceticus. Crude extracts and enzyme fractions containing diaphorases were passed through a Sephadex G-150 columna (33X3 cm). After elution, the collected fractions were assayed as stated in Materials and Methods. A. Crude extract. B. Enzyme fraction I. C. Enzyme fraction II. For experimental details see Materials and Methods. Δ—Δ = Nitrate reductase; ●—● = NADPH-diaphorase; O—O = NADH-diaphorase.

Table I. *Electron acceptors for diaphorases from* Acinetobacter calcoaceticus.
Diaphorase activities were determined as described in Materials and Methods under aerobic conditions except when viologens were used as acceptors.

| Acceptor (mM) | | NAD(P)H oxidized (nmoles/10 min) NADPH-diaphorase I | NADPH-diaphorase II | NADH-diaphorase |
|---|---|---|---|---|
| None | | 0 | 0 | 0 |
| Ferricyanide | 0.3 | 480 | 600 | 960 |
| Menadione | 0.1 | 51 | 141 | 604 |
| DPIP | 0.1 | 230 | 196 | 329 |
| FAD | 0.1 | 6 | 26 | 25 |
| FMN | 0.1 | 6 | 46 | 12 |
| BV | 8 | 70 | 48 | 14 |
| MV | 8 | 34 | 27 | 3 |
| Cytochrome *c* | 0.1 | 0 | 4 | 10 |

be utilized by these diaphorases are shown in table I. In all the cases tested ferricyanide and DPIP were the most effective acceptors. Viologens behaved as good acceptors of the two NADPH-dependent diaphorases whereas menadione and flavin nucleotides exhibited a better effectiveness with respect to NADPH-diaphorase II and NADH-diaphorase. Cytochrome *c* was almost incapable of receiving electrons from NADPH in the diaphorase reaction.

The apparent $K_M$'s values of the diaphorases for NAD(P)H and several electron acceptors are summarized in table II. NICHOLAS and coworkers have previously described a NAD(P)H-viologen reductase in *Azotobacter vinelandii*, but the $K_M$'s values they reported for NADPH and NADH were much higher than those presented here (13-15). The $K_M$ value of NADPH-diaphorase II for DPIP has been calculated by extrapolation from the activity data obtained at low substrate concentration since at high concentrations of DPIP a stimulating effect on the diaphorase activity was observed.

The calculated molecular weights of the three diaphorases, estimated by gel filtration as described in Materials and Methods were: 165,000 for NADPH-diaphorases I and 57,000 for both NADPH-diaphorase II and NADH-diaphorase (fig. 2). A value of 68,000 for a NADH-benzyl viologen reductase from *A. vinelandii* has been reported (13). These sizes deviate from the molecular weight of *Acinetobacter* nitrate reductase, 96,000 (21), and establish a clear difference between diaphorase and nitrate reductase activities in this bacterium in contrast to those of green algae and higher plants (9). So far, no clear participation of any diaphorase activity has been demonstrated in the assimilatory reduction of nitrate carried out by non-photosynthetic bacteria and, in certain cases, reduced ferredoxin seems to be the immediate electron donor for nitrate reduction (3, 19, 20). The question remains whether bacterial diaphorases can transfer reducing power from NAD(P)H to ferredoxin or not. However, since the diaphorases described here are able to reduce both BV and MV (dyes of very low potential, similar to that of ferredoxin) and these reduced dyes are effective donors for the enzymatic reduction of nitrate (21), the participation of these

Table II. *$K_M$'s values of several donors and acceptors for diaphorases from* Acinetobacter calcoaceticus.
$K_M$'s values were calculated from the reciprocal plots of velocity versus concentration of donors and acceptors. Assay conditions are described in Materials and Methods.

| Donor or acceptor | $K_M$ (μM) NADPH-diaphorase I | NADPH-diaphorase II | NADH-diaphorase |
|---|---|---|---|
| NADPH | 34 | 21 | — |
| NADH | — | — | 20 |
| DPIP | 66 | 20 * | 48 |
| Ferricyanide | 214 | 60 | 30 |

* Value obtained by extrapolation discarding the data at high concentrations of DPIP where activation of diaphorase reaction was observed.

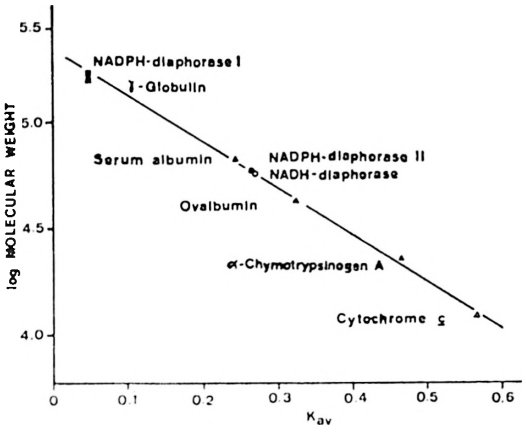



Fig. 2. *Molecular weight of* Acinetobacter *NAD(P)H-viologen diaphorase as determined by gel filtration.*
A Sephadex G-150 column (66.5 × 1.5 cm) was equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 M KCl. The column was calibrated with the indicated standard proteins, and 2 ml fractions were collected. The elution volumen ($V_e$) was calculated by measuring the absorbance of standards at 280 nm, while that of diaphorases by determining their activities as described in Materials and Methods. The void volume ($V_o$) was estimated with Blue Dextran 2000. $V_t$ is the total volume of the gel bed. $K_{av}$ is defined as the ratio $V_e$-$V_o$/$V_t$-$V_o$.

diaphorases in the whole process of bacterial nitrate reduction is a possibility to be ascertained.

The three diaphorase activities were sensitive to thermic inactivation to a different extent. FMN and FAD exerted a marked protective effect against inactivation by heat treatment of NADPH-diaphorase II as well as NADH-diaphorase. This flavin protective effect was absent in NADPH-diaphorase I (table III). Similarly, addition of increasing amounts of exogenous flavin nucleotides showed a linear stimulating effect only on NADPH-diaphorase II and NADH-diaphorase untreated activities (results not shown). It seems, therefore, that flavin coenzymes might play a catalytic role in the activities of NADPH-diaphorase II and NADH-diaphorase from *A. calcoaceticus* as has been shown for many pyridine nucleotide-dependent diaphorases involved in nitrate reduction in other organisms (9).

The diaphorases were differently protected by their own electron donors against heat inactivation. NADH protected effectively NADH-diaphorase whereas NADPH did not protect the NADPH-dependent diaphorases when subjected to the same treatment (table III).

Like the diaphorase activity of the nitrate reductase complex from green algae and higher plants (9), NADPH-diaphorase I was highly sensitive to *p*-HMB, since concentrations as low as $5 \times 10^{-4}$ M inhibited the reaction almost completely, whereas the two other diaphorases were practically unaffected at $10^{-3}$ M concentrations. In this respect, the three diaphorases differ markedly from the *A. calcoaceticus* nitrate reductase which is completely inhibited by *p*-hydroxymercuribenzoate $10^{-5}$ M (20) and from NADH-

Table III. *Effect of flavin and pyridine nucleotides against thermic inactivation of diaphorases from* Acinetobacter calcoaceticus.
The enzymatic preparations were heated in the absence and in the presence of the indicated nucleotides concentration at 55° C, 5 min. Then, they were freed from nucleotides and assayed as described in Materials and Methods. One hundred per cent of activity for NADPH-diaphorase I, NADPH-diaphorase II and NADH-diaphorase, was 123, 102 and 110 nmoles NAD(P)H oxidized/10 min × mg protein, respectively.

| Treatment | Relative specific activity (%) | | |
|---|---|---|---|
| | NADPH-diaphorase I | NADPH-diaphorase II | NADH-diaphorase |
| None | 100 | 100 | 100 |
| Enzyme heated | 10 | 40 | 20 |
| + FAD 50 μM, heated | 7 | 113 | 68 |
| + FMN 50 μM, heated | 8 | 113 | 71 |
| + NADPH 1.5 mM, heated | 10 | 37 | — |
| + NADH 1.5 mM, heated | — | — | 168 |

Table IV. *Effect of reduced pyridine nucleotides on diaphorase activities of* Acinetobacter calcoaceticus.

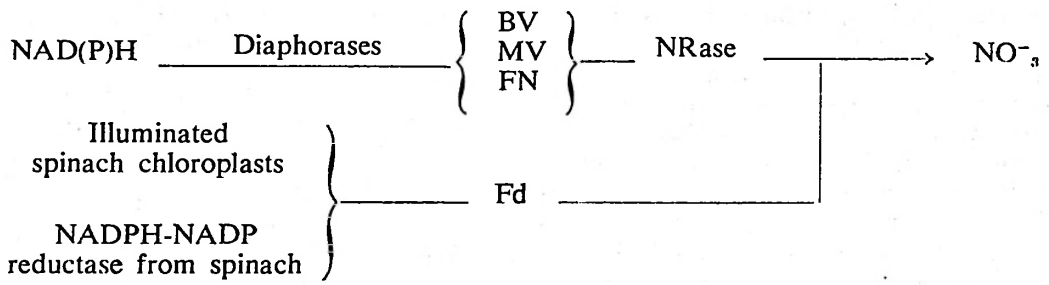
Enzyme preparations containing, in a volume 0.5 ml, 250 μmoles potassium phosphate buffer, pH 7.5, 4 nmoles FAD and 0.3 μmoles of reduced pyridine nucleotides, were incubated for 20 min at room temperature. Then, they were completed up to 2 ml with the remaining components of the assay mixture, and diaphorase activities were determined as indicated in Materials and Methods. One hundred per cent of activity for NADPH-diaphorase I, NADPH-diaphorase II and NADH-diaphorase, was 310, 120 and 570 nmoles NAD(P)H oxidized/5 min × mg protein, respectively.

| | Relative specific activity (%) | | |
|---|---|---|---|
| Treatment | NADPH-diaphorase I | NADPH-diaphorase II | NADH-diaphorase |
| None | 100 | 100 | 100 |
| NADPH | 50 | 220 | — |
| NADH | — | — | 200 |

viologen reductase of *A. vinelandii* which is extremely sensitive to this —SH reagent at a $2 \times 10^{-5}$ M (15). In addition, the three diaphorases were insentive to $5 \times 10^{-4}$ M KCN, a property shared with other diaphorases which participate in nitrate reduction (9).

The diaphorases appear to be subjected to metabolic control since their activities were affected by oxidation-reduction as shown in table IV. NADPH-diaphorase I was inactivated up to 50 % when preincubated with NADPH 1.5 mM for 20 min. Contrarily, NADPH-diaphorase II and NADH-diaphorase showed a great stimulation when subjected to the same treatment in the presence of their respective electron donor. The NADH-viologen reductase from *A. vinelandii* is, however, insensitive to oxidation by potassium ferricyanide or reduction by sodium dithionite (13). In this respect it is worth noting that metabolic control by either NADH or NADPH has been demonstrated for the NAD(P)H-ferredoxin oxidoreductases of several clostridial species (7, 8, 17, 18). The above results point out, therefore, to a clear distinction between the three diaphorases here described.

The present data along with those reported elsewhere (19, 20) suggest the following natural and artificial pathways of electrons for the enzymatic reduction of nitrate in *A. calcoaceticus:*




Whether or not *A. calcoaceticus* ferredoxin can be reduced by NAD(P)H through the diaphorase(s) is a question that needs more investigation. Preliminary attempts to link purified diaphorase(s) system with ferredoxin and nitrate reductase have been until now unsuccessful, possibly due to the fact that some additional factor(s) needed for the coupling of activities might have been lost during purification. However, the existence of ancillary systems capable of transferring electrons from NAD(P)H to ferredoxin as well as the results reported for nitrate reductases of *Clostridium perfringens* (3) and blue-green algae (5, 12, 16), strongly

suggest that nitrate reduction in *A. calcoaceticus* may take place *via* ferredoxin reduced by NAD(P)H through a diaphorase system of the kind described herein.

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## Resumen

Se han aislado y caracterizado parcialmente tres diaforasas dependientes de piridín nucleótidos presentes en células de *Acinetobacter calcoaceticus*. Dos de ellas, de peso molecular 165.000 y 57.000 usan NADPH como donador de electrones y una tercera de peso molecular 57.000, NADH. Los viológenos, las flavinas, diclorofenol indofenol y ferricianuro son buenos aceptores en las reacciones que median estas diaforasas. Las tres actividades se han caracterizado cinéticamente y se ha estudiado el efecto sobre las mismas de diversos inhibidores y cofactores. Estas diaforasas parecen estar sometidas a control metabólico por oxidación y reducción.

## References

1. ANDREWS, P.: *Biochem.*, **91**, 222-233, 1964.
2. CANDAU, P., MANZANO, C. and LOSADA, M.: *Nature*, **262**, 715-717, 1976.
3. CHIBA, S. and ISHIMOTO, M.: *J. Biochem.*, **73**, 1315-1318, 1973.
4. GUERRERO, M. G., VEGA, J. M., LEADBETTER, E. and LOSADA, M.: *Arch. Mikrobiol.*, **91**, 287-304, 1973.
5. HATTORI, A. and MYERS, J.: *Plant Cell Physiol.*, **8**, 327-337, 1967.
6. HEWITT, E. J.: *Ann. Rev. Plant Physiol.*, **26**, 73-100, 1975.
7. JUNGERMANN, K., RUPPRECHT, E., OHRLOFF, C. THAUER, R. K. DECKER, K.: *J. Biol. Chem.*, **246**, 960-963, 1971.
8. JUNGERMANN, K., THAUER, R. K., LEIMENSTOLL, G. and DECKER, K.: *Biochim. Biophys. Acta*, **305**, 268-280, 1973.
9. LOSADA, M.: *J. Mol. Catal.*, **1**, 245-264, 1976.
10. LOWRY, O. H., ROSEBROUGH, M. J., FARR, A. L. and RANDALL, R. J.: *J. Biol. Chem.*, **193**, 265-275, 1951.
11. MALOFEEVA, I. V., NIKONDRATIEVA, E. and RUBIN, A. B.: *FEBS Lett.*, **53**, 188-189, 1975.
12. MANZANO, C., CANDAU, P., GÓMEZ-MORENO, C., RELIMPIO, A. M. and LOSADA, M.: *Mol. Cell Biochem.*, **10**, 161-169, 1976.
13. NAGAI, Y., ELLEWAY, R. F. and NICHOLAS, D. J. D.: *Biochim. Biophys. Acta*, **153**, 766-776, 1968.
14. NAIK, M. S. and NICHOLAS, D. J. D.: *Biochim. Biophys. Acta*, **118**, 195-197, 1966.
15. NAIK, M. S. and NICHOLAS, D. J. D.: *Biochim. Biophys. Acta*, **131**, 204-207, 1967.
16. ORTEGA, T., CASTILLO, F. and CÁRDENAS, J.: *Biochem. Biophys. Res. Commun.*, **71**, 885-891, 1976.
17. PETITDEMANGE, H., CHERRIER, C., RAVAL, G. and GAY, R.: *Biochim. Biophys. Acta*, **421**, 334-347, 1976.
18. THAUER, R. K., RUPPRECHT, E., OHRLOFF, C., JUNGERMANN, K. and DECKER, K.: *J. Biol. Chem.*, **246**, 954-959, 1971.
19. TORTOLERO, M., VILA, R. and PANEQUE, A.: *Plant Sci. Letters*, **5**, 141-145, 1975.
20. VILLALOBO, A.: Doctoral dissertation. Sevilla, 1976.
21. VILLALOBO, A., ROLDÁN, J. M., RIVAS, J. and CÁRDENAS, J.: *Arch. Microbiol.*, **112**, 127-132, 1977.