Vol. 146, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1987 Pages 147-153

INHIBITION STUDIES OF THREE CLASSES OF DESULFOVIBRIO HYDROGENASE : APPLICATION TO THE FURTHER CHARACTERIZATION OF THE MULTIPLE HYDROGENASES FOUND IN DESULFOVIBRIO VULGARIS HILDENBOROUGH

Yves Berlier[1], Guy D. Fauque[1], Jean LeGall[1,2], Eui S. Choi[2], Harry D. Peck Jr[2], and Paul A. Lespinat[1]

[1] Section Enzymologie et Biochimie Bactérienne, ARBS, CEN Cadarache 13108 Saint Paul Lez Durance-Cedex, France

[2] School of Chemical Sciences, Department of Biochemistry, University of Georgia, Athens GA 30602

Received April 23, 1987

The three types of hydrogenase hitherto characterized in genus Desulfovibrio exhibit distinctive inhibition patterns of their proton-deuterium exchange activity by CO, NO and NO_2^-. The (Fe) and (NiFeSe) hydrogenases are the most sensitive to all three inhibitors while the (NiFe) enzymes, relatively little inhibited by CO, are still very sensitive to NO but unaffected by NO_2^-. These differences together with some specific catalytic properties, in particular the pH profile and the H_2 to HD ratio in the exchange reaction, constitute a simple means of characterizing multiple hydrogenases present in one or different species.

Three types of hydrogenase have hitherto been characterized from species of Desulfovibrio. The first type, (Fe), isolated from the periplasm of Desulfovibrio (D.) vulgaris Hildenborough contains exclusively non-heme iron-sulfur centers (1, 2); the second type, (NiFe), represented for instance by the periplasmic hydrogenase from D. gigas includes in addition redox-sensitive nickel (3, 4, 5); the third type, (NiFeSe), first isolated from D. baculatus Norway 4 (6) contains, besides non-heme iron and nickel, selenium atoms possibly substituting for sulfur. The existence of more than one hydrogenase has been recently reported in D. vulgaris Miyazaki F (7) and Hildenborough (8). In addition to the periplasmic

0006-291X/87 $1.50

(Fe) enzyme, two membrane-bound nickel-containing hydrogenases have been isolated from the latter strain (9). One is immunologically related to the soluble (NiFeSe) hydrogenase from D. desulfuricans Norway 4 and the other to the (NiFe) enzyme from D. gigas. The (NiFeSe) hydrogenases have specific properties when compared to the (Fe) and (NiFe) enzymes, such as peculiar patterns in proton-deuterium exchange and in pH profile (10) and a higher activity in H_2 production than in H_2 uptake (11). The present paper will show that, besides these special catalytic properties, the (NiFeSe) hydrogenases also present a specific sensitivity to CO inhibition , intermediate between those of (Fe) and (NiFe) enzymes. NO will prove a very potent inhibitor of the exchange activity in all three types of hydrogenase with again a stronger affinity for (Fe) and (NiFeSe) than for (NiFe) hydrogenases. An inhibitory effect of NO_2^- on the exchange reaction, will also be demonstrated with the (Fe) and (NiFeSe) hydrogenases whereas the (NiFe) type is insensitive.

MATERIALS AND METHODS

The purified enzymes were : the (Fe) periplasmic hydrogenase from D. vulgaris Hildenborough (NCIB 8303) (1, 2); the (NiFe) cytoplasmic hydrogenase from D. multispirans (NCIB 12078) (12) and periplamic hydrogenase from D. gigas (NCIB 9332) (4); the (NiFeSe) periplasmic hydrogenases from D. baculatus (DSM 1743) (13) and D. salexigens British Guiana (NCIB 8403) (14). In addition, the newly isolated (NiFe) and (NiFeSe) membrane-bound hydrogenases from D.vulgaris Hildenborough (9) were also assayed.

Nitrogen (grade N30), carbon monoxide (grade N45) and nitric oxide (grade N30) were from L'Air Liquide. A nitrogen deuterium mixture (80/20, v/v) was prepared from 99.8% deuterium gas purchased from ORIS, Saclay.The chemicals were of analytical grade (Merck or Prolabo).

Hydrogenase activity was measured in the proton-deuterium exchange reaction. It must be stressed that the latter assay avoids the use of redox mediators likely to react chemically with NO and NO_2^- (in particular dithionite). If necessary anaerobiosis can then be ensured by the glucose and glucose-oxidase system. The experiments were performed in a reaction vessel connected via a teflon membrane-inlet to the ion-source of a VG MM8-80 mass-spectrometer monitored by an Apple II data acquisition system (15). The phosphate buffer, pH 6 for D.vulgaris, D. baculatus and D. salexigens or 7.6 for D. gigas and D. multispirans (10) was saturated with the oxygen-free N_2-D_2 mixture before enzyme injection. The exchange reaction was then followed directly on the dissolved gas phase by the appearance of deuterium hydride (HD) and dihydrogen (H_2).

Carbon monoxide and nitric oxide were added as small aliquots of aqueous solutions, respectively 1 and 2 mM, prepared under strict oxygen-free conditions and with the safety precautions relevant to the high toxicity of both gases. Sodium nitrite was injected as small volumes of a deoxygenated solution. The ratio between the exchange activities before and after inhibitor injection,

corrected for the decrease due to deuterium consumption during a similar interval, gave the percentage of inhibition.

RESULTS AND DISCUSSION

In CO inhibition (Fig. 1), the (Fe) periplasmic hydrogenase from D. vulgaris was by far the most sensitive since a 50% decrease was obtained with only 0.1 μM. The (NiFeSe) hydrogenases from D. baculatus, D. salexigens and D. vulgaris (membrane-bound) were also fairly sensitive (about 1 μM of CO for 50% inhibition). The (NiFe) hydrogenases from D. multispirans, D. gigas and D. vulgaris (membrane-bound) were in contrast relatively insensitive since a CO concentration of 20 to 30 μM was required for that same inhibition level. CO inhibition was in all cases reversed by flushing with an inert gas.

It is generally believed that the sensitivity of (Fe) hydrogenases to CO inhibition (16, 17) results from the binding of that gas to the enzyme H_2-activating sites. This is supported by kinetic considerations on the formation and dissociation of an enzyme-CO complex (17) and, more specifically, from EPR and other paramagnetic data. CO was shown to modify a (Fe-S) cluster signal of

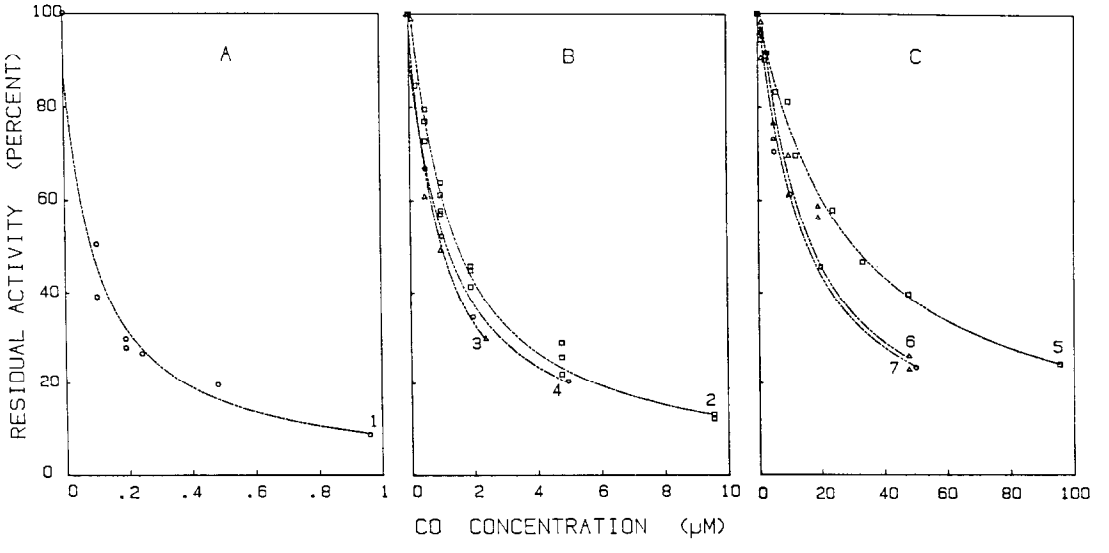

Figure 1. CO inhibition of D_2-H^+ exchange activity in three classes of hydrogenases : A. (Fe) hydrogenase : Curve 1, D. vulgaris (periplasmic) (100% activity = 850 μmol/min/mg) . B. (NiFeSe) hydrogenases : Curve 2, D. baculatus (periplasmic) (220 μmol); Curve 3, D. salexigens (periplasmic) (2100 μmol); Curve 4, D. vulgaris (membrane-bound) (480 μmol). C. (NiFe) hydrogenases : Curve 5, D. gigas (periplasmic) (160 μmol); Curve 6, D. multispirans (cytoplasmic) (600 μmol); Curve 7, D. vulgaris (membrane-bound) (290 μmol).

Table 1. NO inhibition of D_2-H^+ exchange activity in different Desulfovibrio hydrogenases, expressed in percent of full activity (see figure 1)

	(NiFe)			(NiFeSe)	(Fe)
μM NO	D.gigas	D.multispirans	D.vulgaris (membrane)	D.salexigens	D.vulgaris (periplasm)
0.02	/	44	/	93	83
0.04	39	70	/	/	/
0.10	/	89	/	99	99
0.40	96	100	96	100	100

/ not determined

(Fe) hydrogenase from Clostridium (C.) pasteurianum, mainly in the oxidized state (18). The broadening of the ^{13}CO compared to the ^{12}CO signal suggested that a complex was formed between CO and the (Fe-S) cluster (18, 19). Further EPR and ENDOR studies showed the existence in hydrogenase I from that same species of a novel cluster which formed a covalent and irreversible bond with CO (19). In contrast, with the (Fe) hydrogenase from D. vulgaris a reversible CO-binding was demonstrated (20). Although the (NiFe) hydrogenases from D. gigas (21) and Chromatium vinosum (22) are less sensitive to CO inhibition, their nickel moiety was also modified by CO treatment but only in the H_2-reduced enzyme which proved that CO was then binding at the same ligand position as H_2. These studies did not provide evidence that (Fe-S) clusters were modified in the same manner as in (Fe) hydrogenases (22). The molecular basis for a stronger CO-inhibition of (NiFeSe) than of (NiFe) hydrogenases, demonstrated by the present results, has not yet been ascertained by physical techniques.

All three types of enzymes were severely inhibited by very low concentrations of NO. The exchange was almost suppressed with as low as 0.1 μM with the D. vulgaris and D. salexigens periplasmic hydrogenases and with about 0.4 μM with the enzymes from D. multispirans and D. gigas (Table 1). NO inhibition was instantaneous and also fully reversed by sparging with an inert gas. The role of NO as a potent inhibitor of hydrogenase activity was only reported with cell-free extracts of Proteus vulgaris (23) and therefore not necessarily related to the enzyme hydrogenase itself. The only reference to an

Table 2. NO_2^- inhibition of D_2-H^+ exchange activity in (Fe) and (NiFeSe)

	(NiFeSe)		(Fe)
μM NO2-	D.salexigens	D.vulgaris (membrane)	D.vulgaris (periplasm)
0.4	/	/	30
4	18	/	71
20	51	/	/
40	56	/	84
100	73	77	/

/ not determined

Desulfovibrio hydrogenases, expressed in percent of full activity (no inhibition was observed with the (NiFe) hydrogenases).

inhibitory effect of NO upon a purified iron-sulfur enzyme concerns the Fe protein of C. pasteurianum nitrogenase whose (Fe-S) clusters are disrupted by NO (24). This is evidently not the case here since the NO inhibition of hydrogenase exchange activity was fully reversible.

A still more pronounced selectivity was observed towards nitrite inhibition since the (Fe) hydrogenase from D. vulgaris and the (NiFeSe) enzymes from D. salexigens and D. vulgaris (membrane-bound) were inhibited within one or two minutes by NO_2^-, whereas the (NiFe) enzymes from D. gigas, D. multispirans and D. vulgaris (membrane-bound) were unaffected (Table 2).

It must be noted that the (NiFe) hydrogenases, which are insensitive to NO_2^- inhibition, originate from species which harbor a nitrite reductase ammonia-forming activity (25). Some of them like D. multispirans and D. desulfuricans ATCC 27774 are effectively able to respire nitrate instead of sulfate and produce then nitrite as an intermediate in nitrate reduction (26) while the others, like D. gigas, are capable of oxidative phosphorylation in the presence of nitrite (27). The interference of NO_2^- with hydrogenase activity itself was not obvious. The relative slowness of nitrite inhibition on the exchange activity of (Fe) and (NiFeSe) hydrogenases suggests that NO_2^- is probably not an inhibitor per se but has to be transformed to an inhibitory compound. Actually, using the same membrane-inlet technique, a significant NO production (appearance of

mass-peak 30) has been observed with (NiFeSe) hydrogenases in the presence of nitrite and H_2 or D_2.

As a conclusion, there is a striking parallelism in the inhibition patterns of the different hydrogenases by CO, NO and NO_2^-. It is interesting to note here that the general chemical similarities of CO and NO allow them to form similar complexes with transition metals. The higher sensivity to CO of the (Fe) over the (NiFe) hydrogenases is consistent with a different mode of binding between the inhibitor and the active center. Although they contain also nickel, the (NiFeSe) hydrogenases are closer, in their inhibitory pattern, to the (Fe) than to the (NiFe) enzymes. Besides, when compared to most other Desulfovibrio hydrogenases, the (NiFeSe) enzymes exhibit several peculiarities in their catalytic properties, such as a lower pH optimum and higher H_2 to HD ratio in the exchange reaction (10) and a higher activity in H_2 production than in H_2 uptake (11) which prompts to ascribe them to a distinct class.

The proton-deuterium exchange reaction thus provides a simple and specific means of characterizing the different hydrogenases by their catalytic properties and inhibition patterns and, for that particular purpose, can in some instances substitute to the more elaborated spectroscopic techniques which require large amounts of protein. Moreover, in the case of multiple hydrogenases coexisting in the same species as for instance in D. vulgaris, the use of inhibitors can allow the differentiation of their respective physiological role in situ.

ACKNOWLEDGMENTS

We are indebted to B. Dimon and P. Carrier for their skillful help in mass-spectrometric determinations and to B. Prickril, M.H. Czechowski, J.J.G. Moura, I. Moura, A.V. Xavier, S. H. He, and M. Teixeira for providing the purified hydrogenases. This work has been made possible thanks to a special collaborative agreement between the University of Georgia (USA) and the ARBS (France) and was supported in part by the National Science Foundation under contract N° DMB 8602789 and the U.S. Department of Energy under contract N° DEASO9-80-ER10409

REFERENCES

1. Van der Westen, H., Mayhew, S.G. and Veeger, C. (1978) FEBS Lett. 86, 122-126.

2. Huynh, B.H., Czechowski, M.H., Krüger, H.J., DerVartanian, D.V., Peck, H.D.,Jr. and LeGall, J. (1984) Proc. Natl. Acad. Sci. (USA) 81, 3728-3732.
3. Cammack, R., Patil, D., Aguirre, R. and Hatchikian, E.C. (1982) FEBS Lett., 142, 481-484.
4. LeGall, J., Ljungdahl, P.O., Moura, I., Peck, H.D.,Jr, Xavier, A.V., Moura, J.J.G., Teixeira, M., Huynh, B.H. and DerVartanian, D.V. (1982) Biochem. Biophys. Res. Commun. 106, 610-616.
5. Teixeira, M., Moura, I., Xavier, A.V., Huynh, B.H., DerVartanian, D.V., Peck, H.D. Jr, Le Gall, J. and Moura, J.J.G. (1985) J. Biol. Chem., 1960, 8942-8950.
6. Rieder, R., Cammack, R. and Hall, D.O. (1984) Eur. J. Biochem. 145, 637-643 .
7. Yagi, T., Kimura, K. and Inokuchi, H. (1985) J. Biochem., 97, 181-187.
8. Gow, L.A., Pankhania, I.P., Ballantine, S.P., Boxer, D.H. and Hamilton, W.A. (1986) Biochim. Biophys. Acta 851, 57-64.
9. Lissolo, T., Choi, E.S., LeGall, J. and Peck, H.D.,Jr. (1986) Biochem. Biophys. Res. Commun. 139, 701-708.
10. Lespinat, P.A., Berlier, Y., Fauque, G., Czechowski, M., Dimon, B. and LeGall, J. (1986) Biochimie 68, 55-61.
11. Fauque, G.D., Berlier, Y.M., Czechowski, M.H, Dimon B., Lespinat, P.A. and LeGall, J. (1987) J. Ind. Microbiol., in the press.
12. Czechowski, M.H., He, S.H., Nacro, M., DerVartanian, D.V., Peck, H.D.,Jr. and LeGall, J. (1984) Biochem. Biophys. Res. Commun. 108, 1388-1393.
13. Teixeira, M., Moura, I., Xavier, A.V., Moura, J.J.G., Fauque, G., Prickril, B. and LeGall, J. (1985) Rev. Port. Quim. 27, 194-195.
14. Teixeira, M., Moura, I., Fauque, G., Czechowski,M., Berlier, Y., Lespinat, P.A., LeGall, J., Xavier, A.V. and Moura, J.J.G. (1986) Biochimie 68, 75-84.
15. Berlier, Y., Dimon., B., Fauque, G. and Lespinat, P.A. (1985) in "Gas Enzymology", (Degn, H., Cox, R.P. and Toftlund, H., Eds), pp. 17-35, Reidel Publishing Company, The Hague.
16. Adams, M.W.W. and Mortenson, L.E. (1984) J. Biol. Chem. 259, 7045-7055.
17. Thauer, R.K., Käufer, B., Zähringer, M. and Jungermann, K. (1974) Eur. J. Biochem. 42 447-452.
18. Erbes, D.L., Burris, R.H. and Orme-Johnson, W.H. (1975) Proc. Natl. Acad. Sci. (USA) 72, 4795-4799.
19. Telser, J., Benecky, M.J., Adams, M.W.W., Mortenson, L.E. and Hoffman, B.M. (1986) J. Biol. Chem. 261, 13536-13541.
20. Patil, D.S., Czechowski, M.H., Huynh, B.H., LeGall, J., Peck, H.D., Jr. and DerVartanian,.D.V. (1986) Biochem. Biophys. Res. Commun. 137, 1086-1093.
21. DerVartanian, D.V., Krüger, H.J., Peck, H.D,Jr. and LeGall, J. (1985) Rev. Port. Quim. 27, 70-73.
22. Van der Zwaan, J.W., Albracht, S.P.J., Fontijn, R.D. and Roelofs, Y.B.M. (1986) Biochim. Biophys. Acta 872, 208-215.
23. Krasna, A.I. and Rittenberg, D. (1954) Proc. Natl. Acad. Sci. (USA) 40, 225-227.
24. Meyer, J. (1981) Arch. Biochem. Biophys. 210, 246-256.
25. Mitchell, G.J., Jones, J.G. and Cole, J.A. (1986) Arch. Microbiol. 144, 35-40.
26. Steenkamp, D.J. and Peck, H.D.,Jr. (1980) Biochem. Biophys. Res. Commun. 94, 41-48.
27. Barton, L.L., Le Gall, J., Odom, J.M. and Peck, H.D. Jr (1983) J. Bacteriol., 154, 867-871.