Carbamoylphosphate requirement for synthesis of the active center of [NiFe]-hydrogenases

Athanasios Paschos[a], Richard S. Glass[b], August Böck[a,*]

[a]*Institute of Genetics and Microbiology, University of Munich, Maria-Ward-Straße 1a, 80638 Munich, Germany*
[b]*Department of Chemistry, University of Arizona, P.O. Box 210041, Tucson, AZ 85721-0041, USA*

Received 6 December 2000; accepted 13 December 2000

First published online 22 December 2000

Edited by Barry Halliwell

Abstract The iron of the binuclear active center of [NiFe]-hydrogenases carries two CN and one CO ligands which are thought to confer to the metal a low oxidation and/or spin state essential for activity. Based on the observation that one of the seven auxiliary proteins required for the synthesis and insertion of the [NiFe] cluster contains a sequence motif characteristic of *O*-carbamoyl-transferases it was discovered that carbamoyl phosphate is essential for formation of active [NiFe]-hydrogenases in vivo and is specifically required for metal center synthesis suggesting that it is the source of the CO and CN ligands. A chemical path for conversion of a carbamoyl group into cyano and carbonyl moieties is postulated

Key words: [NiFe]-Hydrogenase; Metal center formation; Carbamoylphosphate; Cyano/carbonyl ligand

1. Introduction

Elemental hydrogen plays a central role in the metabolism of many microorganisms. It is produced in order to remove a surplus of reducing power via the reduction of protons or it serves as an energy source by oxidation into two protons and two electrons. The enzymes involved are the hydrogenases which consist of two major classes, the Fe-only hydrogenases ([Fe]-hydrogenases) and the [NiFe]-hydrogenases (for review see [1]).

The catalytic center of both types of enzymes has been analyzed to a considerable detail by X-ray diffraction [2,3] and Fourier transform infrared spectroscopy [4,5]. In [Fe]-hydrogenases it consists of a [4Fe–4S] cluster linked to a 2Fe cluster via the thiolate of a cysteine residue from the protein backbone. The two Fe centers in the 2Fe cluster are bridged via a propanedithiol molecule and are substituted by one carbonyl and one cyano group each [6]. The catalytic center of [NiFe]-hydrogenases consists of an Fe substituted with two cyano and one carbonyl and a Ni liganded by four thiolates of cysteine residues from the protein, two of which bridge the Ni with the Fe site, [2–4].

Whereas assembly of the active site of [Fe]-hydrogenases has been little investigated there is considerable information on the metallocenter synthesis of [NiFe]-hydrogenases. Apart from the two structural genes encoding the large and small subunits, the products of seven genes were identified to serve as auxiliary proteins to generate an active enzyme [7]. These are the products of the six so-called *hyp* genes (HypA, HypB, HypC, HypD, HypE and HypF) and that of another gene encoding a specific endopeptidase. The present conjecture is that the two metals are inserted separately, first the Fe with its CO and CN ligands, assisted by the binding of a specific chaperone, HypC, to the large subunit [8,9]. Nickel is supplied by the HypB protein which possesses GTPase activity required for the assembly process [10]. The specific endopeptidase then scans the apoprotein of the large subunit for the presence of the correct metal and removes a C-terminal short peptide; this cleavage triggers a conformational switch which leads to internalization of the catalytic center [8,11].

A unique feature of both [Fe]-hydrogenases and [NiFe]-hydrogenases is that CO and CN groups are ligands of the Fe. It is neither known how they are synthesized nor how they are attached to the metal. The resolution of this open question is intriguing not only because CN and CO are among to the most toxic chemicals in biology but also because it presents an unprecedented problem in bioinorganic chemistry. In the present communication we show that carbamoylphosphate is required for the maturation of [NiFe]-hydrogenases and chemical routes are suggested how carbamoylphosphate can be converted into both the cyano and carbonyl ligands.

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli strains MC4100 [12] and P4X [13] were used as wild type strains. JEF8 is a derivative of P4X carrying a deletion in the *carB* gene [14]. Strain ΔcarAB lacks a major part of the *carAB* operon; it was derived from MC4100 during the course of this work (see below). DHPF is an MC4100 decendent carrying an in-frame deletion in the *hypF* gene [15]. Plasmid pCarAB is derived from pBR322 [16]; it contains an insert consisting of the *carAB* operon plus 200 bp of the flanking region on each side bordered by *Hin*dIII and *Bam*HI restriction sites. The insert was generated by PCR on chromosomal DNA of *E. coli* MC4100 using the oligonucleotides *carAB*/*Hin*dII (5′-CCCCCAAGCTTGTCGCTTAATGCCTGTAAAACA-3′) and *carAB*/*Bam*HI (5′-CGCGGATCCATATTAAAATTGTGGATATATCGA-3′). Plasmid pCarAB was converted into plasmid pΔCarAB by removal of a 3530 bp *Eco*RV/*Cfr*91 fragment. The deletion generated reaches from nucleotide position 336 of the *carA* gene to position 2700 of the *carB* gene. The *Hin*dIII/*Bam*HI fragment from it carrying the deletion was cloned into plasmid pMAK700 [17] to yield plasmid pMΔCarAB. pMΔCarAB was transferred into *E. coli* MC4100 and the integration into the chromosome by homologous recombination was conducted according to Hamilton et al. [17]. The authenticity of the chromosomal in-frame deletion was confirmed by DNA sequencing.

*Corresponding author. Fax: (49)-89-2180 2122.
E-mail: august.boeck@lrz.uni-muenchen.de

0014-5793/01/$20.00
PII: S0014-5793(00)02408-X

2.2. Media and growth conditions

For plasmid isolation and transformation purposes, bacteria were grown in LB medium [18]. Anaerobic cultivation was performed either in a buffered rich medium (TGYEP) [19] or in minimal medium [20] which were fortified with 1 μM sodium molybdate, 1 μM sodium selenite, 5 μM nickel chloride and, where indicated, with 30 mM sodium formate. Methionine and threonine were supplemented at 100 μg/ml final concentration and uracil, citrulline and arginine were added at the concentrations indicated.

Maintenance of plasmids was selected for by inclusion of antibiotics into the medium, namely ampicillin at 100 μg/ml and chloramphenicol at 30 μg/ml.

2.3. Preparation of cell-free extracts and determination of hydrogenase activity

The *E. coli* strains were grown under anaerobic conditions and at 37°C to an OD_{600} of 1,0. They were harvested by centrifugation and resuspended in 10 mM Tris–HCl (pH 7,4) containing 20 μg/ml phenylmethylsulfonyl fluoride and 20 μg/ml desoxyribonuclease I. The cells were broken by two consecutive passages through a French Press cell at 118 MPa and the extract was centrifuged at $10\,000 \times g$ for 30 min.

The hydrogenase activity of the extracts was determined by H_2-dependent benzyl viologen reduction following the method of Ballantine and Boxer [21].

Alternatively, samples of the crude extracts were separated by non-denaturing polyacrylamide electrophoresis at 100 V for 4 h and at 4°C. The gels were then incubated in 100 mM sodium phosphate buffer (pH 7.2) containing 0.5 mM benzyl viologen and 1 mM tetrazolium chloride under an atmosphere of 96% CO_2 and 4% H_2 for 24 or 48 h.

3. Results and discussion

A clue on how CO and CN synthesis may proceed emerged recently from the observation that one of the accessory gene products, HypF, possesses a sequence motif (VXHHXAH) which also is present in proteins catalysing *O*-carbamoylations during synthesis of antibiotics and nodulation factors [22–24] (our unpublished results). It has been pointed out previously that HypF also contains two identical zinc finger motifs ($CX_2CX_{18}CX_2C$) [25] and an acyl phosphatase motif [26]. Since the carbamoyl group appeared to be a suitable adduct for the synthesis of either the carbonyl or of the cyano group or both of them we have analyzed whether carbamoylphosphate is required for the synthesis of active [NiFe]-hydrogenases in vivo. For this purpose, a deletion was introduced into the *carAB* operon from *E. coli* MC4100 which removed most of the two reading frames. The resulting strain, ΔCarAB was grown in the rich buffered medium TGYEP and crude extracts were prepared and analyzed for hydrogenase activity. Fig. 1 shows that the deletion abolishes the ability of the mutant to synthesize active hydrogenases. When transformed with a plasmid expressing the *carAB* transcriptional unit the capacity for formation of active hydrogenase was restored.

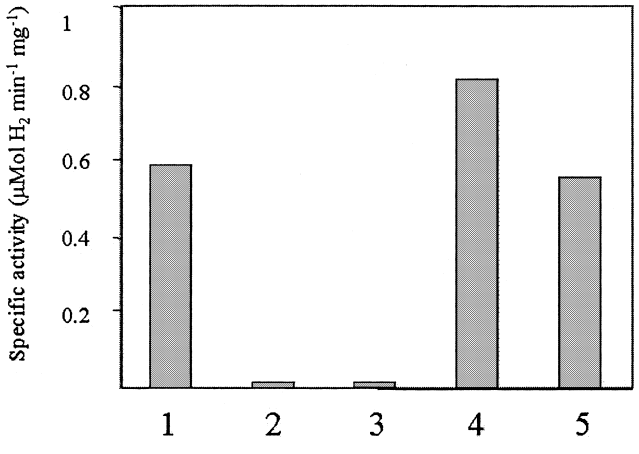


Fig. 1. Specific hydrogenase activity of crude extracts of fermentatively grown cells from *E. coli* strains (1) MC4100, (2) DHPF, (3) ΔCarAB, (4) ΔCarAB/pCarAB, (5) ΔCarAB grown in the presence of 200 μg/ml L-citrulline. The medium was TGYEP, the temperature 37°C. Results are the average of three independent experiments.

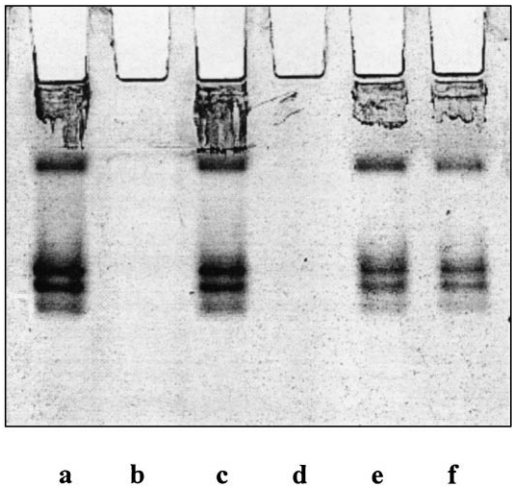


Fig. 2. Requirement in vivo of carbamoylphosphate for synthesis of active hydrogenases 1 and 2 from *E. coli*. The strains indicated were grown fermentatively in minimal medium containing 0.8% glucose plus the required supplements and cellular extracts were prepared, 100 μg protein were subjected to polyacrylamide gel electrophoresis under non-denaturing conditions and the gels were stained for hydrogenase activity [21]. Lanes: (a, b) Wild type MC4100 and its *ΔhypF* derivative; (c, d) wild type P4X and its *ΔcarB* derivative JEF8 grown in the presence of 50 μg/ml L-arginine and uracil, each (e) JEF8 cells transformed with a plasmid expressing the *carAB* genes grown in the absence of arginine and uracil; (f) JEF8 cells grown in the presence of 50 μg/ml L-arginine plus uracil, each plus 200 μg/ml L-citrulline. The migration positions of hydrogenase 1 (three conformers) and of hydrogenase 2 are indicated.

Since carbamoylphosphate can be generated at low rate from citrulline by the reversal of the anabolic ornithine transcarbamoylase reaction it was tested whether citrulline may suffice in the generation of hydrogenase activity. When added at concentrations of 200 μg/ml or higher this was indeed the case. As already shown previously [15], a derivative of *E. coli* MC4100 with an in-frame deletion in *hypF* was completely devoid of hydrogenase activity.

E. coli is able to synthesize at least three active NiFe hydrogenases. If carbamoylphosphate is the source of the CO and CN ligands the activity of all three isoenzymes should be affected in a mutant unable to synthesize it. To test this assumption mutant JEF8 (*met*, *thre*, *carB*) which is a derivative of *E. coli* P4X [13] and carries a mutation in *carB*, the gene for the catalytic subunit of carbamoylphosphate synthetase was grown fermentatively in glucose minimal medium supplemented with the required amino acids plus arginine and uracil, the crude extracts were separated by non-denaturing polyacrylamide electrophoresis and the gel was subjected to substrate staining (Fig. 2). From the three hydrogenases of *E. coli* [7] isoenzymes 1 and 2 can be resolved; isoenzyme 3 cannot be detected under this condition. Identical results were obtained with mutant ΔCarAB (results not shown).

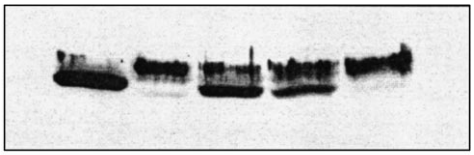
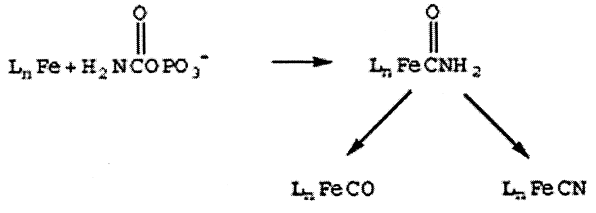


Fig. 3. Maturation of the large subunit of hydrogenase 3 from *E. coli*. An immunoblot of an SDS polyacrylamide gel is shown developed with antibodies directed against the large subunit of hydrogenase 3. Lanes: (a) Wild type MC4100, (b) JEF8 grown in the presence of 50 μg/ml L-arginine and uracil each, (c) JEF8/pCarAB grown in the absence of arginine and uracil, (d) JEF8 grown in the presence of 200 μg/ml L-citrulline and 50 μg/ml of L-arginine and uracil, each; (e) MC4100 *ΔhypF*.

In comparison to the two wild type strains MC4100 (lane a) and P4X (lane c), extracts from JEF8 cells were devoid of hydrogenase 1 and 2 activity (lane d) as well as those cells having a deletion in the *hypF* gene (lane b). Transformation of JEF8 with a plasmid carrying the *carAB* operon restored the activity of the two hydrogenases (lane e) as did supplementation of the medium with citrulline at 200 μg/ml (lane f). Thus carbamoylphosphate is an essential metabolite for the synthesis of active [NiFe]-hydrogenases.

A crucial experiment then was to prove that carbamoylphosphate is required for synthesis of the metal cluster and not for some other reaction. One way to achieve this is to analyze whether the proteolytic processing of the large subunit, which is a measure for the correct incorporation of both $Fe(CO)(CN)_2$ and Ni, occurs. Thus, cells grown under identical conditions as described in the experiment of Fig. 2 were tested for cleavage of the precursor of the large subunit of hydrogenase 3 (Fig. 3). Extracts from DHP-F (*ΔhypF*) and JEF8 (*carB*) have unprocessed large subunit in comparison to the wild type; growth in the presence of 200 μg per ml citrulline or transformation of JEF8 with a plasmid carrying *carAB* restored processing. It is thus clear that carbamoylphosphate is required for synthesis of the metal cluster. Since CO and CN are the only organic constituents of the metal center the results support the contention that carbamoylphosphate is the precursor either for CO or CN alone or for both.

Further support comes from the fact that metal carbamoyl complexes can be converted into both metal cyano and metal carbonyl complexes. Conversion of carbamoyl phosphate to carbonyl and cyano ligands on iron presents some novel organometallic chemistry as shown in Eq. 1.



(1)

Nucleophilic displacement on the carbon of carbamoyl phosphate by iron, resulting in a carbamoyl iron complex, is a likely initial step. Metal carbamoyl complexes are known [27–29] but have not yet been extensively studied. Low-valent anionic iron complexes such as $Fe(CO)_4^{2-}$ (Collman's reagent) and $(\pi\text{-}C_5H_5)Fe(CO)_2^-$ are known [30,31] to be excellent nucleophiles toward alkyl and acyl halides. Iron carbamoyl complexes are also formed by the reaction of $(\pi\text{-}C_5H_5)Fe(CO)_2^-$ with carbamoyl chlorides [32]. However, similar such species are unlikely biological intermediates. Since nucleophilic displacement involves formally oxidative addition at iron, three mechanistic possibilities need to be considered. Formation of an Fe(I) species by reduction followed by nucleophilic displacement results in an Fe(III) species. This sequence is comparable to alkylation of reactions of Co(I) formed by reduction of Vitamin B_{12} to Vitamin B_{12s} [33]. Alternatively, nucleophilic displacement with Fe(II) generates an Fe(IV) intermediate stabilized by thiolate ligands which are good σ- and π-electron donors. High-valent iron species are formed in the reactions of cytochrome P-450 [34]. Notably the iron center in this enzyme is ligated to the thiolate of a cysteine residue. A third possibility is that nucleophilic displacement by Fe (II) occurs concomitantly with ligand oxidation by one or two electrons. For example, the two thiolate ligands donate two electrons to the iron resulting in carbamoyl Fe (II) and disulfide. Subsequent reduction of the disulfide regenerates the two thiolate ligands.

Transformation of the carbamoyl iron moiety into a carbonyl iron species is analogous to the known [35] reaction of carbamoyl manganese (I) complexes to give the corresponding carbonyl metal complexes on treatment with HCl as shown in Eq. 2.

$$Mn(CO)_4\,(CONHR)\,(NH_2R) + 2HCl \rightarrow [Mn(CO)_5\,(NH_2R)]^+\,Cl^- + RNH_3^+\,Cl^- \quad (2)$$

Conversion of a carbamoyl group attached to iron into a cyano group, as shown in Eq. 1, is analogous to the known [36] dehydration of manganese carbamoyl complexes to cyano complexes and related to the known [37] transformation of carbamoyl into isocyanide ligands on iron as illustrated in Eq. 3.

$$(\pi - C_5H_5)\,(CO)_2\,FeCONHCH_3 + COCl_2 + Et_3N \rightarrow [(\pi - C_5H_5)(CO)_2\,FeCNCH_3]^+\,Cl^- + CO_2 + Et_3NH^+\,Cl^- \quad (3)$$

A reasonable hypothesis for converting a carbamoyl ligand into a cyano group under biological conditions is the following. Protonation of the oxygen of the carbamoyl group, which is analogous to the known [38,39] *O*-alkylation of iron acylate complexes with hard nucleophiles, generates formally a cationic metal carbene complex. Dehydration of this intermediate results in a cationic metal complex of isocyanic acid. Metal complexes of isocyanides are well-known [40] but those of isocyanic acid are rare [41]. Loss of a proton generates the metal cyano species. Thus conversion of a carbamoyl ligand on iron into a carbonyl or cyano group depends on whether the nitrogen or oxygen of the ligand is protonated. The site of protonation should depend on the oxidation state and ligands on the iron.

The results presented prove that carbamoyl phosphate is required for formation of the [NiFe] metallocenter of hydrogenases and they strongly support the contention that CO and CN are derived from it. It will be interesting to

elucidate whether the organometallic precedents are followed in the biological system and to see whether carbamoylation of Fe directly takes place in the large subunit or whether it occurs at some scaffold protein followed by transfer to the large subunit.

Acknowledgements: A.B. and R.S.G. thank the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie for support.

References

[1] Adams, M.W.W. and Stiefel, E.L. (1998) Science 282, 1842–1843.
[2] Volbeda, A., Charon, M.H.M., Piras, C., Hatchikian, E.C., Frey, M. and Fontecilla-Camps, J.C. (1995) Nature 373, 580–587.
[3] Higuchi, Y., Yagi, T. and Yasuoka, N. (1997) Structure 5, 1671–1680.
[4] Bagley, K.A., Duin, E.C., Roseboom, W., Albracht, S.P.J. and Woodruff, W.H. (1995) Biochemistry 34, 5527–5535.
[5] Happe, R.P., Roseboom, W., Pierik, A.J., Albracht, S.P.J. and Bagley, K.A. (1997) Nature 385, 126.
[6] Peters, J.W., Lanzilotta, W.N., Lemon, B.J. and Seefeldt, L.C. (1998) Science 282, 1853–1858.
[7] Drapal, N., Maier, T.H.P. and Böck, A. (1998) Period Biol. 100, 45–51.
[8] Drapal, N. and Böck, A. (1998) Biochemistry 37, 2941–2948.
[9] Magalon, A. and Böck, A. (2000) J. Biol. Chem. 275, 21114–21120.
[10] Maier, T., Lottspeich, F. and Böck, A. (1995) Eur. J. Biochem. 2, 133–138.
[11] Fritsche, E., Paschos, A., Beisel, H.G., Böck, A. and Huber, R. (1999) J. Mol. Biol. 288, 989–998.
[12] Casadaban, M.J. and Cohen, S.N. (1979) Proc. Natl. Acad. Sci. USA 76, 4530–4533.
[13] Graham, A., Jenkins, H.E., Smith, N.H., Mandrand-Berthelot, M.A., Haddock, B.A. and Boxer, D.H. (1980) FEBS Lett. 113, 167–172.
[14] Mergeay, D., Gigot, J., Beckmann, N., Glansdorff, N. and Pierard, A. (1974) Mol. Gen. Genet. 133, 299–316.
[15] Maier, T., Binder, U. and Böck, A. (1996) Arch. Microbiol. 165, 333–341.
[16] Balbas, P., Soberon, X., Meriono, E., Zurita, M., Lomeli, H., Valle, F., Flores, N. and Bolivar, T. (1986) Gene 50, 3–40.
[17] Hamilton, C.M., Aldea, M., Washburn, B.K., Babitzke, P. and Kushner, S.R. (1989) J. Bacteriol. 171, 4617–4622.
[18] Miller, J.H. (1992) A short course in bacterial genetics, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY.
[19] Begg, Y.A., Whyte, J.N. and Haddock, B.A. (1997) FEMS Microbiol. Lett. 2, 47–50.
[20] Fraenkel, D.G. and Neidhardt, F.C. (1961) Biochim. Biophys. Acta 53, 96–100.
[21] Ballantine, S.P. and Boxer, D.H. (1985) J. Bacteriol. 163, 454–459.
[22] Mao, Y., Varoglu, M. and Sherman, D.H. (1999) Chem. Biol. 6, 251–263.
[23] Steffensky, M., Muhlenweg, A., Wang, Z.X., Li, S.M. and Heide, L. (2000) Antimicrob. Agents Chemother. 44, 1214–1222.
[24] Freiberg, C., Fellay, R., Bairoch, A., Broughton, W.J., Rosental, A. and Perret, X. (1997) Nature 387, 394–401.
[25] Yamamoto, T., Tomiyama, M., Mita, H., Sode, K. and Karube, I. (1990) FEMS Microbiol. Lett. 66, 187–192.
[26] Wolf, I., Buhrke, T., Dernedde, J., Pohlmann, J. and Friedrich, B. (1998) Arch. Microbiol. 170, 451–459.
[27] Angelici, R.J. (1972) Acc. Chem. Res. 5, 335–341.
[28] Ford, C. and Rokicki, A. (1988) Adv. Organomet. Chem. 28, 139–217.
[29] Anderson, S., Hill, A.F. and Ng, Y.T. (2000) Organometallics 19 (and references therein), 15–21.
[30] Collman, J.P. (1975) Acc. Chem. Res. 8, 342–347.
[31] Pearson, R.G. and Figdore, P.E. (1980) J. Am. Chem. Soc. 102, 1541–1547.
[32] King, R.B. (1963) J. Am. Chem. Soc. 85, 1918–1922.
[33] Schrauzer, G.N., Deutsch, E. and Windgassen, R.I. (1968) J. Am. Chem. Soc. 90, 2441–2442.
[34] Ortiz de Montellano P.R., Cytochrome P 450: Structure, Mechanism and Biochemistry, Plenum, New York, 1995.
[35] Angelici, R.J. and Denton, D.L. (1968) Inorg. Chim. Acta 2, 3–7.
[36] Behrens, H., Lindner, E., Maertens, D., Wild, P. and Lampe, R.-T. (1972) J. Organomet Chem. 34, 367–377.
[37] Fehlhammer, W.P. and Mayr, A. (1975) Angew. Chem. 87, 776–777.
[38] Semmelhack, M.F. and Tamura, R. (1983) J. Am. Chem. Soc. 105, 4099–4100.
[39] Fischer, E.O., Schneider, T. and Ackerman, K. (1984) Z. Naturforsch. 39b, 468–473.
[40] Singleton, E. and Oosthuizen, H.E. (1983) Adv. Organomet. Chem. 22, 209–310.
[41] Bruce, M.I. and Wallis, R.C. (1981) Aust. J. Chem. 34, 209–213.