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Homologous and Heterologous Overexpression in *Clostridium acetobutylicum* and Characterization of Purified Clostridial and Algal Fe-Only Hydrogenases with High Specific Activities

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***Clostridium acetobutylicum* ATCC 824 was selected for the homologous overexpression of its Fe-only hydrogenase and for the heterologous expressions of the *Chlamydomonas reinhardtii* and *Scenedesmus obliquus* HydA1 Fe-only hydrogenases. The three *Strep* tag II-tagged Fe-only hydrogenases were isolated with high specific activities by two-step column chromatography. The purified algal hydrogenases evolve hydrogen with rates of around 700 μmol H_2 min^{-1} mg^{-1}, while HydA from *C. acetobutylicum* ($HydA_{Ca}$) shows the highest activity (5,522 μmol H_2 min^{-1} mg^{-1}) in the direction of hydrogen uptake. Further, kinetic parameters and substrate specificity were reported. An electron paramagnetic resonance (EPR) analysis of the thionin-oxidized $HydA_{Ca}$ protein indicates a characteristic rhombic EPR signal that is typical for the oxidized H cluster of Fe-only hydrogenases.**

The great biotechnological interest of hydrogen is its use as a fuel, an alternative "clean" energy carrier. Hydrogenases catalyze the interconversion of hydrogen gas (H_2) and its elementary particle constituents, protons and electrons. They are a diverse group of enzymes, often classified according to the transition metal cofactors associated with the protein (25). Among the hydrogenases, the Fe-only hydrogenases often catalyze the reduction of protons to yield hydrogen at high turnover levels (2, 17, 19).

To our knowledge, the fastest reported microorganism for hydrogen production from hexose is the anaerobic bacterium *Clostridium acetobutylicum*, with a productivity in a chemostat of 2.4 liters H_2 $liter^{-1}$ h^{-1} (22). *C. acetobutylicum* ATCC 824 contains an Fe-only hydrogenase (12) and a putative NiFe-hydrogenase revealed by genome sequencing (18). The Fe-only hydrogenase gene is located on the chromosome, whereas the NiFe-hydrogenase is located on a separate megaplasmid. Only one report described the purification of the *C. acetobutylicum* Fe-hydrogenase related to its ability to reduce 2,4,6-trinitrotoluene (27), but nothing yet is known on the respective involvement of NiFe- and Fe-hydrogenases in the hydrogen metabolism of *C. acetobutylicum*.

Hydrogen evolution from light and water by green algal Fe-hydrogenases is an active field of basic and applied research. *Chlamydomonas reinhardtii* contains two Fe-hydrogenases (HydA1 and HydA2). HydA1 is located in the chloroplast and linked to the photosynthetic electron transport chain via the ferredoxin PetF (9, 13, 14). Two copies of Fe-hydrogenase genes were also identified in the green alga *Scenedesmus obliquus* (7, 29). All these algal Fe-hydrogenases have transcripts expressed upon anaerobic induction (7, 9, 13). The "classical" isolation of native *C. reinhardtii* and *S. obliquus* Fe-only hydrogenases revealed highly active hydrogenases showing hydrogen evolution activities above 700 μmol H_2 min^{-1} mg^{-1} (7, 13, 14). Those Fe-hydrogenases from algae and clostridia reveal common features, e.g., monocistronic gene organization, a monomeric structure (between 44.5 and 64 kDa), and high homology at the amino acid sequence level in the C-terminal region including the H catalytic center (7, 25). However, algal hydrogenases lack an additional Fe-S cluster in the N-terminal domain (25).

To develop an efficient hydrogen production system, the study of structure-function relationships of Fe-hydrogenases is needed, requiring the characterization of overexpressed purified native and modified Fe-hydrogenases. Since the heterologous expression of Fe-only hydrogenases in *Escherichia coli* or in the cyanobacterium *Synechococcus* sp. led to enzymes in an inactive form (5, 12, 26) or with low hydrogen evolution activity (4, 20), we developed a homologous and heterologous overexpression and purification system functional in *C. acetobutylicum*, an essential tool for the characterization of Fe-hydrogenases.

Development of an overexpression and purification system functional in *C. acetobutylicum*. Our system is based on (i) *E. coli*-*C. acetobutylicum* shuttle vectors for overexpression in *C. acetobutylicum* and (ii) a fusion protein technique for a rapid and efficient purification procedure. The development of this

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TABLE 1. Bacterial strain and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
C. acetobutylicum ATCC 824	Wild type	ATCC[b]
Plasmids		
pMFH1	*hydA*$_{Ca}$ gene, ORF2, ORF3, Amp^r, ColE1 origin	12
pPHhydA$_{Ca}$-C-tag	*hydA*$_{Ca}$ promoter, *hydA*$_{Ca}$ gene, *Strep* tag II, Ap^r, Em^r, *repL* gene, ColE1 origin	This study
pSOS95[c]	*thlA* promoter, acetone operon, Amp^r, Ery^r, *repL* gene, ColE1 origin	8
pThydA$_{Ca}$-C-tag	*thlA* promoter, *hydA*$_{Ca}$ gene, *Strep* tag II, Amp^r, Ery^r, *repL* gene, ColE1 origin	This study
pThydA$_{Ca}$-N-tag	*thlA* promoter, *Strep* tag II, *PS* sequence, *hydA*$_{Ca}$ gene, Amp^r, Ery^r, *repL* gene, ColE1 origin	This study
pThydA$_{Crl}$-C-tag	*thlA* promoter, *Strep* tag II, *PS* sequence, *hydA*$_{Crl}$ gene, Amp^r, Ery^r, *repL* gene, ColE1 origin	This study
pThydA$_{Sol}$-C-tag	*thlA* promoter, *Strep* tag II, *PS* sequence, *hydA*$_{Sol}$ gene, Amp^r, Ery^r, *repL* gene, ColE1 origin	This study

[a] Abbreviations: Amp^r, ampicillin resistance; Ery^r, erythromycin resistance; *repL* gene, gram-positive origin of replication from pIM13; *thlA* promoter, promoter of the *C. acetobutylicum* ATCC 824 thiolase gene (23); *hydA*$_{Ca}$ gene, *C. acetobutylicum* ATCC 824 [Fe]-hydrogenase gene; *hydA*$_{Crl}$ gene, *C. reinhardtii* HydA1 [Fe]-hydrogenase gene; *hydA*$_{Sol}$ gene, *S. obliquus* HydA1 [Fe]-hydrogenase gene; *PS* sequence, cleavage recognition site of the prescission protease (Amersham).

[b] ATCC, American Type Culture Collection Manassas, Va.

[c] GenBank accession number AY187686.

system was performed with the *C. acetobutylicum* Fe-hydrogenase (HydA$_{Ca}$). Since first attempts using glutathione *S*-transferase protein led mainly to an insoluble fusion protein, the small tag *Strep* tag II, consisting of only eight amino acids, was evaluated. This tag was reported to be efficient in metalloenzyme purification (15). *Strep* tag II was positioned in the N-terminal (pThydA$_{Ca}$-N-tag vector) or C-terminal (pThydA$_{Ca}$-C-tag vector) position of HydA$_{Ca}$, under control of the ribosome binding site (RBS) and promoter (P$_{thlA}$) from clostridial thiolase (Table 1 and Fig. 1). All plasmids were constructed in *E. coli*. The *hydA*$_{Ca}$ gene was sequenced in pThydA$_{Ca}$-N-tag and pThydA$_{Ca}$-C-tag before transfer into *C. acetobutylicum* (16). *C. acetobutylicum* recombinant strains were stored in spore form at −20°C, being stable for months.

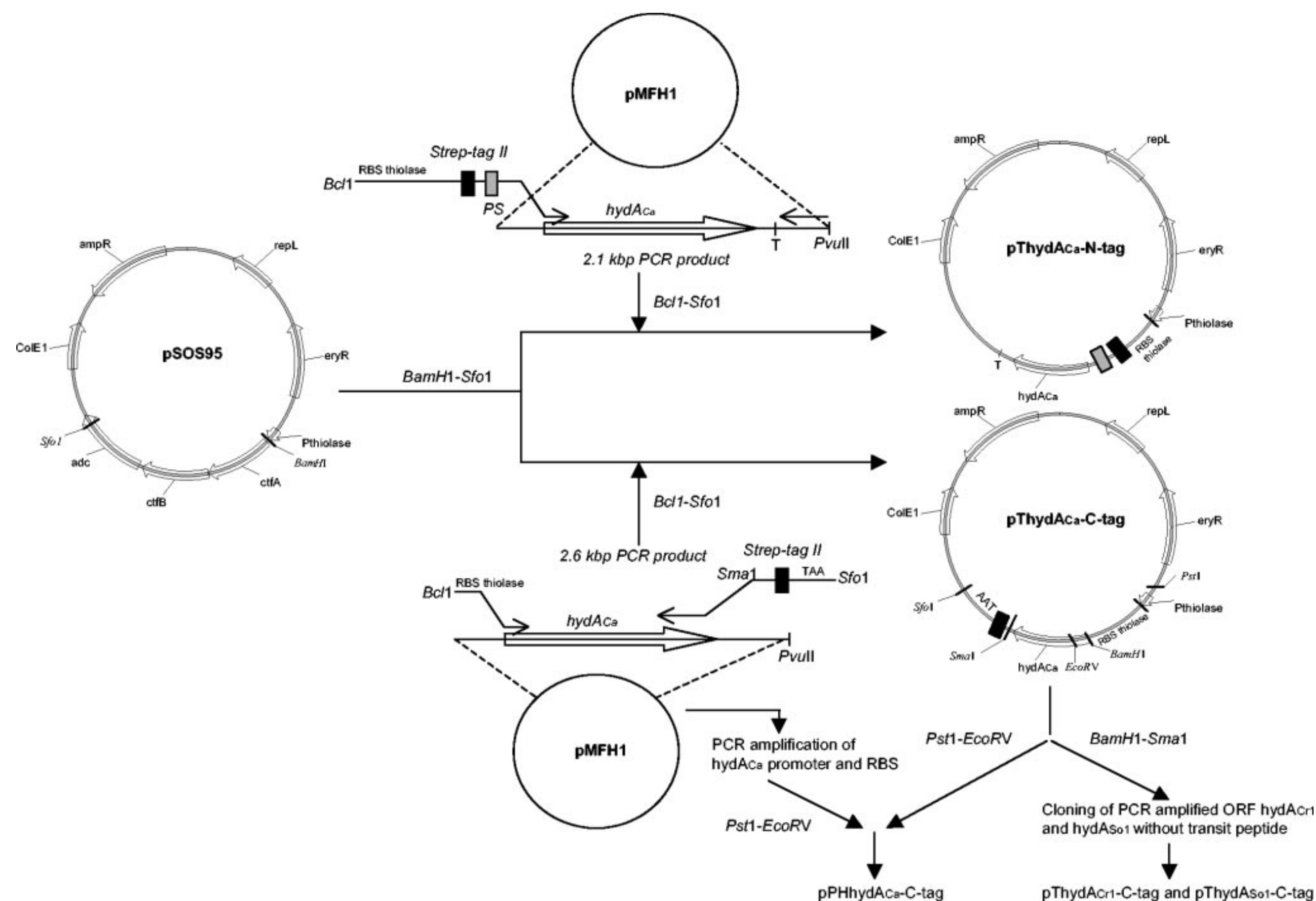

FIG. 1. Construction of the shuttle expression vectors. For each plasmid, the locations and directions of relevant genes and restriction sites are shown. *adc* and *ctfAB* are genes encoding *C. acetobutylicum* ATCC 824 acetoacetate decarboxylase and coenzyme A transferase in the acetone operon. T, *hydA*$_{Ca}$ terminator. For definitions of other abbreviations, see the footnotes to Table 1.

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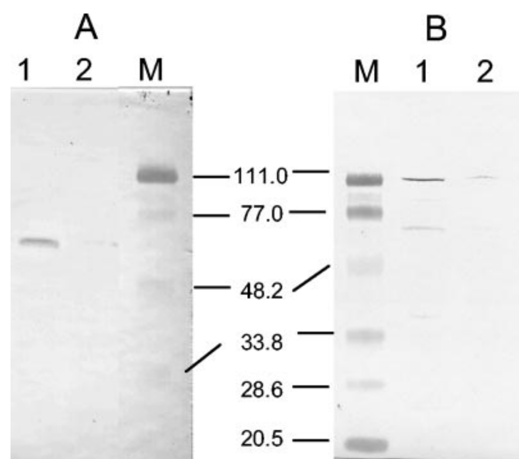

FIG. 2. Immunoblot detection with Strep Tactin HPR conjugate (IBA GmbH, Göttingen, Germany) of *Strep* tag II-HydA$_{Ca}$ in sonicated *C. acetobutylicum* cells with *Strep* tag II in the N-terminal position (A) (lane 1, postsonication pellet; lane 2, cell extract) and *Strep* tag II in the C-terminal position (B) (lane 1, cell extract; lane 2, postsonication pellet). M, prestained low-range standard proteins (sizes are in kDa).

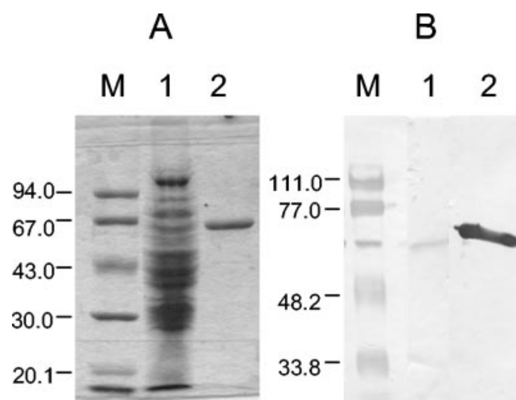

FIG. 3. Purification of *Strep* tag II-HydA$_{Ca}$. (A) Coomassie staining. Lane 1, crude *C. acetobutylicum* cell extract; lane 2, purified *Strep* tag II-HydA$_{Ca}$ protein (~2 μg). (B) Immunoblot detection with Strep Tactin HPR conjugate. Lane 1, crude *C. acetobutylicum* cell extract; lane 2, purified *Strep* tag II-HydA$_{Ca}$ protein (~2 μg). M, low-range standard proteins (sizes are in kDa).

Strep tag II-HydA$_{Ca}$ (64 kDa) was overexpressed in *C. acetobutylicum*. The tagged protein's solubility was greatly influenced by the tag position. After sonication, N-terminal *Strep* tag II-HydA$_{Ca}$ was mostly found in the postsonication pellet while C-terminal *Strep* tag II-HydA$_{Ca}$ was exclusively detected in the cell extract (Fig. 2). The C-terminal *Strep* tag II-HydA$_{Ca}$ construct was selected for the following optimization experiments.

The expression level of recombinant HydA$_{Ca}$ in *C. acetobutylicum* was increased after exchanging in the expression vector the P_{thlA} promoter for the 14 times stronger *hydA*$_{Ca}$ promoter (P_{hydA}) as previously demonstrated in cultures maintained at pH 6.5 (10). Consequently, P_{hydA}, together with the Fe-only hydrogenase RBS, was cloned in place of the thiolase promoter and RBS into the pThydA$_{Ca}$-C-tag vector to yield pPHhydA$_{Ca}$-C-tag. The level of *Strep* tag II-HydA$_{Ca}$ expression was compared between cell extracts of *C. acetobutylicum* strains pThydA$_{Ca}$-C-tag and pPHhydA$_{Ca}$-C-tag grown in batch cultures at a pH regulated at 6.5. For both recombinant strains, *Strep* tag II-HydA$_{Ca}$ expression was constant throughout the exponential phase. However, for the same culture volume, 10 times more *Strep* tag II-HydA$_{Ca}$ could be purified with the same level of specific activity using *C. acetobutylicum* pPHhydA$_{Ca}$-C-tag compared with *C. acetobutylicum* pThydA$_{Ca}$-C-tag (data not shown).

Homologous overexpression, purification, and characterization of the *C. acetobutylicum* Fe-only hydrogenase. Anaerobic conditions were maintained throughout the entire expression-purification procedure in the presence of 2 mM Na-dithionite. *C. acetobutylicum* pPHhydA$_{Ca}$-C-tag was grown on a minimum medium (24) supplemented with erythromycin (40 μg ml^{-1}), calcium carbonate (2 g liter^{-1}), and a mixed solution of iron sulfate (100 mg liter^{-1}), nickel II chloride (6 mg liter^{-1}), zinc sulfate (120 mg liter^{-1}), and nitrilotriacetic acid (400 mg liter^{-1}) in a 1.3-liter batch culture maintained at 37°C and pH 6.5, the optimal pH of P_{hydA} expression (10). While cells were still in the exponential growth phase (optical density at 620 nm in the range of 5 to 6), they were anaerobically harvested (15 min at 9,000 rpm and 4°C), transferred into the anaerobic chamber, washed in 100 mM Tris/HCl (pH 7.6)–2 mM Na-dithionite–10% glycerol, concentrated 30 times, and frozen at −20°C. The cell yield was around 1.5 to 2 g liter^{-1}. Later, again in the anaerobic chamber, the frozen cells were thawed and broken by sonication and debris was removed by centrifugation (10 min at 6,000 rpm). Nucleic acids were precipitated by addition of streptomycin sulfate (2 g liter^{-1}) in the supernatant and removed by centrifugation. A first rough protein separation was performed in batch by anionic chromatography on 2 ml Q-Sepharose (Amersham) in 25 mM Tris/HCl–2 mM Na-dithionite at pH 8.3. Elution occurred stepwise, eluting *Strep* tag II-HydA$_{Ca}$ in the 0.3 M NaCl fraction. The second purification step was affinity chromatography on a 1-ml Strep-Tactin Superflow column performed as described by the manufacturer (IBA GmbH, Göttingen, Germany). *Strep* tag II-HydA$_{Ca}$ was eluted by gravity in pure form in a single step by competition with 2.5 mM desthiobiotin (Fig. 3). An accurate purification factor could not be calculated since the native hydrogenases separated from the recombinant hydrogenase only on the second column. From a 1-liter culture we could routinely obtain 0.4 mg of recombinant *Strep* tag II-HydA$_{Ca}$ protein, a yield sufficiently high to get enough protein for biophysical characterizations.

For electron paramagnetic resonance (EPR) analysis, a 100-μl sample of the isolated *Strep* tag II-HydA$_{Ca}$ protein (10 mg ml^{-1}) was oxidized by anaerobic direct addition of 3,7-diamino-5-phenothiazinium (thionin, >85%; Sigma) to a final concentration of 20 μM. *C. pasteurianum* Cp1 Fe-hydrogenase was prepared in exactly the same manner as the *Strep* tag II-HydA$_{Ca}$ protein. The samples were then anaerobically transferred to a quartz EPR tube (Wilmad, Buena, NJ) and immediately frozen in liquid nitrogen. X-band EPR spectroscopy was performed using a Bruker EMX spectrometer equipped with an ER 4119HS high-sensitivity cavity and an Oxford Instruments ESR-900 helium flow cryostat. The EPR spectrum was recorded at 15 K, a microwave power of 2 mW,

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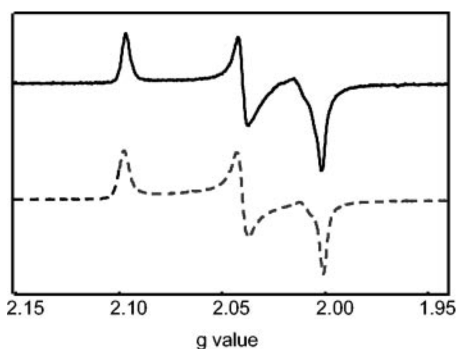

FIG. 4. X-band EPR spectrum of thionin-oxidized *Strep* tag II-$HydA_{Ca}$ protein (solid line). For comparison, X-band EPR spectrum of thionin-oxidized Cp1 protein is shown (dashed line). Spectroscopic parameters are detailed in the text.

a modulation amplitude of 10.0 G, a modulation frequency of 100 kHz, a sweep rate of 100 Gs^{-1}, and a microwave frequency of 9.84 GHz. An independently recorded background spectrum of the cavity and a thionin contaminant was aligned with and subtracted from the experimental spectrum to ensure that the final generated spectrum was solely derived from the paramagnetic component of the *Strep* tag II-$HydA_{Ca}$ protein. The X-band EPR spectrum of thionin-oxidized *Strep* tag II-$HydA_{Ca}$ protein is presented in Fig. 4. The thionin-oxidized *Strep* tag II-$HydA_{Ca}$ protein exhibited a characteristic rhombic EPR signal due to a single paramagnetic species. The signal is due to the oxidized H cluster of the protein, which exhibits an $S = 1/2$ paramagnetic ground state with the line shape and g values (2.096, 2.039, and 2.00) of the spectrum being in close agreement with those previously reported for other oxidized Fe-only hydrogenases (Fig. 4 and references 1, 3, and 6). The lack of any significant perturbations to the typical oxidized EPR signal of the *Strep* tag II-$HydA_{Ca}$ protein suggests the electronic properties of the H cluster within the protein have not been affected by the addition of the *Strep* tag II tag.

To measure purified $HydA_{Ca}$ activities, the in vitro hydrogen uptake assay was adopted from Vasconcelos et al. (24) while the in vitro hydrogen evolution activity was measured as previously described (11). The hydrogen uptake and evolution activities were 5,522 and 10 μmol H_2 min^{-1} mg^{-1}, respectively, using methylviologen. These activities are significantly lower than those of *C. pasteurianum* Fe-only hydrogenase Cp1—4.3 and 340 times lower for the hydrogen uptake and evolution activities, respectively (2, 19)—and also lower than activities of the native and recombinant green algal hydrogenases (7, 14, and next section).

For hydrogen uptake activity, the turnover (k_{cat}) was 60 times higher with methylviologen while an almost 100-fold higher catalytic efficiency (k_{cat}/K_m) was measured for ferredoxin compared to methylviologen as electron acceptor (Table 2). For hydrogen evolution activity, both k_{cat} and k_{cat}/K_m were higher for reduced ferredoxin than for reduced methylviologen, 5 and 250 times, respectively (Table 2). Obviously, the natural substrate ferredoxin was the more suitable substrate for efficient hydrogen uptake and hydrogen evolution activities of *C. acetobutylicum* Fe-only hydrogenase. For comparison, Quinkal and coworkers (21) have reported a slightly higher affinity of Cp1 for reduced ferredoxin associated with a 50 times higher V_{max}.

Heterologous expression and characterization of recombinant HydA from green algae. Fe-only hydrogenases from the green algae *C. reinhardtii* and *S. obliquus* were expressed in *C. acetobutylicum* using the expression system described above. The cDNA coding region was cloned without the 5′ region encoding the N-terminal transit peptide of the *hydA1* genes from *C. reinhardtii* and *S. obliquus* (Fig. 1). The inserts of $pThydA_{Cr1}$-C-tag and $pThydA_{So1}$-C-tag were sequenced, confirming that the fragments contain the exact full coding region of the algal hydrogenase without transit peptide.

Expression and purification occurred from *C. acetobutylicum* transformants containing the plasmids $pThydA_{Cr1}$-C-tag and $pThydA_{So1}$-C-tag. *C. acetobutylicum* cells were grown in complex CGM medium (28) in 1.3-liter batch cultures at a constant pH of 6.5. Similar expression and purification steps as described for homologous hydrogenase from *C. acetobutylicum* were performed for the algal hydrogenases. Due to the high oxygen sensitivity of the enzymes, high concentrations of Na-dithionite (10 mM) had to be maintained throughout the whole procedure. During the stepwise elution from Q-Sepharose, the algal hydrogenases were eluted at 0.5 M NaCl. After purification using the StrepTactin Superflow column, about 0.1 mg algal hydrogenase was isolated from a 1-liter culture (Fig. 5). Although the amount of HydA is not high, similar values were obtained for HydA isolated from algal cultures of *C. reinhardtii* and *S. obliquus* (7, 13, 14). It should be noted that the thiolase promoter (P_{thlA}) was used in this study and that by using the hydrogenase promoter (P_{hydA}) the expression level could be increased significantly.

The isolated Fe-only hydrogenases were highly stable under anaerobic conditions. At 30 μg/ml, recombinant algal hydrogenase and *Strep* tag II-$HydA_{Ca}$ had lost 25 and 40% of their activity, respectively, when stored for 7 days in the absence of oxygen at room temperature kept in 0.1 M Tris/HCl (pH 8.0)–10 mM Na-dithionite.

Hydrogen evolution rates of 760 and 633 μmol H_2 min^{-1} mg^{-1} and K_m values of 508 and 386 μM for $HydA_{Cr1}$ and $HydA_{So1}$, respectively, were measured with methylviologen as electron donor. These kinetic parameters were in the same range as described earlier in the literature (7, 14) for the native algal and clostridial Fe-only hydrogenases. The hydrogen evo-

TABLE 2. Kinetic parameters of $HydA_{Ca}$ for methylviologen and ferredoxin as electron acceptor or donor

Electron acceptor or donor	K_m (M)	V_{max} (μmol min^{-1} mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Oxidized methylviologen	128×10^{-3}	10,057	10,793	8.4×10^4
Oxidized ferredoxin	23×10^{-6}	160	172	7.5×10^6
Reduced methylviologen	0.3×10^{-3}	10	11	3.6×10^4
Reduced ferredoxin	6×10^{-6}	51	55	9.1×10^6

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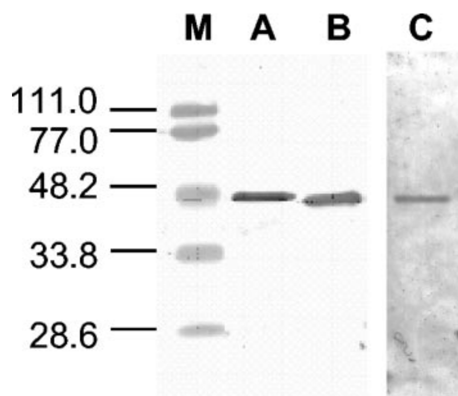

FIG. 5. Purification of $HydA_{Cr1}$. Immunoblot detection probed with Strep Tactin HPR conjugate (A), $HydA_{Cr1}$ antibody (B), or Ponceau red staining (C). M, low-range standard proteins (sizes are in kDa). Protein concentration, ~2 μg.

lution rate of $HydA_{Cr1}$ expressed in *C. acetobutylicum* was significantly higher than the 0.4 μmol H_2 min^{-1} mg^{-1} reported when the enzyme was expressed in *E. coli* (20). Due to high Na-dithionite concentrations in the buffer of purified protein, hydrogen consumption activity tests could not be carried out in a quantitative manner.

Interestingly, the same H_2 production rates were determined using clostridial ferredoxin as electron donor (data not shown). This indicates that the algal hydrogenases accept not only plant-type ferredoxin with a [2Fe-2S] cluster but also [4Fe-4S] bacterial-type ferredoxin. It is known that [2Fe-2S] plant-type ferredoxins are efficient electron mediators to clostridial hydrogenases (25).

In summary, the *Strep* tag II–Fe-only hydrogenase of *C. acetobutylicum* has been purified. In comparison with Cp1, the electronic properties of its H cluster were similar but its hydrogen evolution activity was strongly reduced. The heterologously expressed *C. reinhardtii* and *S. obliquus* Fe-only hydrogenases were fully active with high specific activities. This overexpression and purification system represents a major breakthrough for the study of the structure-function relationships of Fe-only hydrogenases.

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