J Ind Microbiol Biotechnol (2006) 33: 368–376
DOI 10.1007/s10295-005-0067-y

ORIGINAL PAPER

Razia Kutty · George N. Bennett

Studies on inhibition of transformation of 2,4,6-trinitrotoluene catalyzed by Fe-only hydrogenase from *Clostridium acetobutylicum*

Received: 11 April 2005 / Accepted: 3 December 2005 / Published online: 28 January 2006

Abstract The major enzyme in *Clostridium acetobutylicum* ATCC 824 leading to transformation of TNT has been reported to be the Fe-only hydrogenase. In this study, we examine the effect of inhibitors of hydrogenase on TNT reduction by Clostridial extracts. These experiments further demonstrate the major role of hydrogenase in TNT transformation. The *C. acetobutylicum* hydrogenase is closely related to that of *C. pasteurianum;* and can be fitted to the X-ray crystal structure with a root mean square deviation of 1.18 Å for the Cα atoms of the generated 3D simulation model. The Hyd1, Hyd2, and Hyd3 antibodies generated against hydrogenase reacted with both the hydrogenase in cell extracts and with *C. acetobutylicum* hydrogenase expressed in *Escherichia coli*. Inhibition studies using antibodies against Fe-only hydrogenase from *C. acetobutylicum* indicated that the transformation of TNT by crude cell extracts was completely inhibited by Hyd2 antibody (to amino acid 415–428) whereas antibodies Hyd1 (to residues 1–16) and Hyd3 (to amino acid 424–448) inhibited less effectively. The TNT transforming activity of the cell extract was retained when Hyd2 antibody pretreated with purified but enzymatically inactive recombinant hydrogenase was added to the extract. Addition of the transition metal Cu (2+) to extracts completely inhibited the transformation of TNT suggesting the destruction of [4Fe–4S] centers which are essential for transfer of electrons from the H2-activating site to TNT. Growth of *C. acetobutylicum* was also inhibited by 0.5 mM Cu(2+) and Hg(2+) ions. The triazine dye, procion red and the nitroimidazole drug, metronidazole inhibit TNT reduction. The inhibition studies using antibodies, procion red, metronidazole, and transition metals suggest that different portions of hydrogenase are required for effective TNT reduction.

R. Kutty · G. N. Bennett (✉)
Department of Biochemistry and Cell Biology MS-140,
Rice University, Houston, TX 77005-1892, USA
E-mail: gbennett@rice.edu
Tel.: +1-713-3484920
Fax: +1-713-3485154

Keywords Reduction · Hydrogen · TNT · Antibody · Nitroaromatic

Introduction

Nitroaromatic compounds are released into the biosphere almost exclusively from man-made sources [20, 36]. Most of the nitroaromatics are highly recalcitrant compounds. Some of these nitro-compounds are produced by incomplete combustion of fossil fuels; others are used as synthetic intermediates, dyes, pesticides, and explosives. Trinitrotoluene (TNT) is a major contaminant at various Department of Defense facilities. Due to concerns regarding toxicity and environmental health effects of TNT and its reduced metabolites [7, 13, 20] current research has focused on remediation by biological processes which are also cost effective compared to physicochemical methods [43].

Our previous studies have demonstrated that a major activity responsible for TNT transformation in *C. acetobutylicum* ATCC 824 is Fe-only hydrogenase [47]. This was shown by protein purification and experiments with genetically modified strains. Hydrogenases are a heterogeneous group of enzymes that differ in molecular composition, specific activity in catalyzing H_2 production and H_2 oxidation, electron carrier specificity, cofactor content, and sensitivity to inactivation by O_2 [2]. However they have a common feature, all contain highly reducing inorganic iron–sulfur centers that allow them to participate in electron transfer reactions at extremely low redox potentials [1]. The hydrogenases are categorized into two classes: the Fe-only hydrogenases and the Ni–Fe hydrogenases on the basis of active site metals. The Ni–Fe hydrogenases are the most commonly found hydrogenases in sulfate reducing bacteria. These hydrogenases contain a nickel–iron sulfur center which is believed to be the site at which hydrogen binds, and additional iron–sulfur clusters that probably serve as secondary electron carriers [9].

The 3D structures for the monomeric Fe-only hydrogenase from *Clostridium pasteurianum* and periplasmic heterodimeric Ni–Fe hydrogenase from *Desulfovibrio gigas* have been determined [38, 46]. *C. pasteurianum* hydrogenase belongs to Fe-only hydrogenase class and contains five iron–sulfur clusters, one of which is termed the H-cluster and is the center of catalytic activity [9].

The structure of dimeric periplasmic Fe-only hydrogenase from *Desulfovibrio desulfuricans* has also been solved and the features of di-iron active site center, the plausible electron and proton transfer pathways and a putative channel for access of hydrogen to the active site have been identified [34]. The amino acids that line the hydrophobic channel running from the molecule's surface to the active site in the *D. desulfuricans* structure are highly conserved with *C. pasteurianum* hydrogenase suggests a similar internal pathway for either the uptake or the production of molecular hydrogen. This channel is also similar to those observed in the *Desulfovibrio fructosovorans* Ni–Fe hydrogenase [32]. These channels are probably involved to facilitate hydrogen access to the active site in this class of enzyme [33]. The overall structure of *D. desulfuricans* and *C. pasteurianum* are structurally similar and are divided into an active site domain and accessory cluster domains [37].

During nitro group reduction hydrogenases interact with ferredoxins that have low redox potential and can be reduced by other oxidoreductases [3]. The interaction of ferredoxins with several redox enzymes is determined by electrostatic interactions within the complex formed [17]. Studies modeling the charge distribution on the surface structure of *C. pasteurianum* hydrogenase indicate the surface is mostly negatively charged with the exception of one positively charged region which is close to the [4Fe–4S] cluster FS4C which could attract the negative charged surface area of ferredoxin [23]. The structure–function studies with ferredoxins have demonstrated that complexes between ferredoxin and its redox partners do not depend on localized interactions involving a small number of residues but make use of the shape complementarity between large areas of the whole molecules [16, 29, 33, 42].

In this study the involvement of the catalytic domains and the Fe–S centers in the reactions of TNT transformation by Fe-only hydrogenase enzyme has been investigated. These experiments were done in extracts to examine the contribution of Fe-only hydrogenase to total TNT reducing activity and to observe if other enzymes (e.g., nitroreductases and other redox enzymes) may also contribute. The sequence of the *Clostridium acetobutylicum* ATCC 824 genome (http://www.jura.ebi.ac.uk:8765/ext-genequiz/genomes/ca0108/) indicates the presence of genes encoding nitroreductase family proteins, which have sequence similarity to oxygen insensitive nitroreductases purified from aerobic and facultative bacteria. Angermaier et al. [5] have shown that the nitroreductase activity of cell extracts of *Clostridium kluyveri* was NADH dependent and attributed the activity to the redox enzymes NADH ferredoxin oxidoreductase or butyryl-CoA dehydrogenase.

The amino acid sequence of Fe-only hydrogenase of *C. acetobutylicum* shows 84% similarity to that of the Fe-only hydrogenase from *C. pasteurianum* and hence could be structurally identical. A 3D simulation model was made for the hydrogenase of *C. acetobutylicum* based on sequence homology with the *C. pasteurianum* hydrogenase reported in literature [38]. Antibodies against conserved amino acid sequence motifs of the Fe-only hydrogenase of *C. acetobutylicum* were prepared. The reduction of nitroimidazole drug metronidazole by hydrogenase and the effect of various inhibitors on hydrogenase activity have been extensively investigated [11, 27, 28, 30]. Inhibitory experiments of TNT reduction in the presence of antibodies, metronidazole, triazine dye, procion red, and transition metal ions can implicate possible sites on the hydrogenase enzyme in its reaction with TNT.

Materials and methods

Chemicals

All chemicals used for media preparation were reagent grade unless otherwise noted. Solid chemicals used include 2,4,6-trinitrotoluene (99% purity) was procured from Chem Service, West Chester, PA, USA. The solvent used, acetonitrile (99.9%, Fisher Scientific, Pittsburgh, PA, USA), was HPLC grade.

Bacterial growth conditions

Cultures of *Escherichia coli* BL21 DE3 harboring the expression plasmid pET41a (Novagen) were grown at 37°C in Luria-Bertani medium (LB) containing 30 μg/ml of kanamycin as the selection antibiotic. Cultures of *C. acetobutylicum* were grown at 37°C on Clostridial growth medium (CGM) pH 7, prepared as described by Hartmanis and Gatenbeck [21] in an anaerobic chamber (Forma Scientific, Marietta, OH, USA) under an atmosphere of 85% N_2, 9.9% H_2, and 5.1% CO_2. *C. acetobutylicum* strains were stored as corn mash spores or as lyophilized cells in glass ampoules.

Cell extract preparation

Clostridia cultures were harvested after 18 h of growth by centrifugation (16,000×*g* for 10 min at 4°C). The cell extract was prepared anaerobically as described previously [26]. The Bradford assay method (BioRad, Philadelphia, PA, USA) was used to determine protein content of the extracts using bovine serum albumin as the standard protein.

Analytical techniques

The TNT concentrations were measured as described in Huang et al. [25] with the following modification: analytes were separated on a reverse-phase Waters Nova-Pak-C18 column (2×150 mm) at room temperature with gradient mobile phases consisting of water/acetonitrile (75/25 to 5/15 [vol/vol]) at 0.25 ml/min.

Antibodies to hydrogenase

The *hydA* gene (CAC0028) of *C. acetobutylicum* ATCC 824 was aligned against closely related species (*D. desulfuricans*, *C. pasteurianum*, *Clostridium saccharobutylicum*, *Clostridium perfringens*, *D. fructosovorans*, *Clostridium thermocellum*) by the clustal W program and three conserved sequence motifs were selected viz. Hyd1 (1–16 amino acid residues), Hyd2 (415–428), and Hyd3 (424–441 residues) for raising antibodies. The numbering refers to the amino acid sequence of the Fe-only hydrogenase enzyme of *C. acetobutylicum*. The peptides were synthesized and antibodies against these peptides were prepared by Alpha Diagnostics International (San Antonio, TX, USA). Nonspecific antibodies were removed from the antisera by absorption on nontarget antigens from a *C. acetobutylicum* cell extract immobilized on nitrocellulose membranes following the negative purification method [6]. SDS-PAGE electrophoresis of cell extracts was carried out by loading 150 μg of protein in each lane followed by transferring the protein to a nitrocellulose membrane and the band corresponding to that of hydrogenase was identified by staining with acidic ponceau S (0.1% ponceau S in 5% acetic acid) and excised. The membrane was then incubated with 10 ml of antibodies (1:10) in a tray overnight at 4°C. This treatment of antibody was repeated until the nonspecific background signal on Western blot analysis was eliminated**.** The treated antibody was further used for in vitro inhibition experiments.

Western blot analysis

Cell extracts were prepared from *C. acetobutylicum* ATCC 824 by the protocol described previously [26] and analyzed by Western blotting. One-dimensional denaturing SDS-PAGE (12% polyacrylamide) was followed by electrophoretic transfer to Biotrace NT nitrocellulose membrane (Gelman Sciences, Ann Arbor, MI, USA). The resulting membranes were probed with negatively purified Hyd1, Hyd2, and Hyd3 antibodies (1:1,000) followed by a 1:1,000 titer of secondary antibody (Goat anti-rabbit IgG) conjugated with horseradish peroxidase (Pierce, Rockford, IL, USA). Visualization was via diaminobenzidene/chloronapthol staining (Pierce).

Recombinant hydrogenase expression in *E. coli*

The *hydA* gene (CAC 0028) was amplified by PCR using primers, forward: GGTCTCNCATG AAAACAATAATC, reverse: CGCGGATCCTTATTCATGTTTTGAAAC containing flanking NcoI and BamHI sites (underlined). The recognition sequence BsaI (underlined) upstream of NcoI site in the forward primer was used to generate NcoI compatible overhangs. The PCR product was cloned into the corresponding sites in the pET41a expression vector (Novagen, Madison, WI, USA) location producing pET41a-hyd. *E .coli* strain BL21 (DE3) was transformed with pET41a-hyd. Expression was induced with 1 mM isopropyl-*β*-D-thiogalactoside. Purification was carried out by glutathione affinity column (GST bind kit, Novagen) according to the supplier's protocol. The purified protein of 67 kDa was obtained after digesting the fusion protein (20 μg) with 0.5 units of enterokinase (Novagen) at room temperature for 3 h. The reaction mixture was mixed with GSH–STI resin (1:1) mixture of 50% glutathione agarose resin (Stratagene, La Jolla, CA, USA) and 50% soybean trypsin inhibitor resin (Stratagene) to remove undigested GST fusion protein, GST tag, and added enterokinase. The purified hydrogenase was recovered in the flow through.

In vitro inhibition studies of TNT transformation

Differential inhibition of TNT reduction was carried out with antibodies against three peptides Hyd1 (amino acids 1–16), Hyd2 (amino acids 415–428), and Hyd3 (amino acids 424–448). *C. acetobutylicum* ATCC 824 cell extracts (100 μg protein) were treated with hydrogenase antibodies Hyd1, Hyd2, Hyd3 (1:10 titer) in 5 mM Tris buffer pH 7.8 at 4°C overnight. TNT reduction assays were carried out under anaerobic conditions in 1 atm. of H_2. TNT (100 μM) transformation by the treated extract was monitored by HPLC at 1.5, 2.5, and 3.5 h. In parallel, controls were maintained with no antibody, preimmune sera, chemical control (TNT in 5 mM Tris pH 7.8), and biological controls (cell extract was first boiled and then incubated with TNT). The purified recombinant hydrogenase after fusion protein removal was used for the inhibition studies. The recombinant hydrogenase (100 μg) was inactive towards TNT transformation. The Hyd2 antibody (1:10 titer) pretreated overnight at 4°C with inactive recombinant hydrogenase (100 μg) was used as an antibody control to examine TNT transformation by cell extracts (100 μg) in 5 mM Tris buffer pH 7.8.

Inhibition studies

The TNT reduction was monitored in the presence of different concentrations of metronidazole (0.1, 0.2, and

0.5 mM) in *C. acetobutylicum* cell extracts. The reactions contained cell extract (100 μg) and TNT (100 μM) in 5 mM Tris pH 7.8 with different concentrations of metronidazole or procion red dye. Samples were removed after 60-min intervals. Cell extracts (100 μg) were incubated with 0.5 mM transition metal salts for 5 min followed by addition of TNT (100 μM). Samples were subsequently removed for TNT transformation analysis after 30 min incubation. To examine the inhibitory effect of the transition metal ions [Cu(2+), Hg(2+), Ni(2+), and Co(2+)] on the growth of *C. acetobutylicum;* stock solutions of metal salts were added to the CGM media to give the desired metal concentration.

Three-dimensional simulation model

The hydrogenase of *C. acetobutylicum* has sequence similarity of 84% with that of *C. pasteurianum* hydrogenase [31]. A 3D simulation model was made for hydrogenase of *C. acetobutylicum* by using the geno3D protein modeling software [12]. The structure was superimposed by a Unix based graphic program [Ribbons, University of Alabama at Birmingham (UAB) Center for Macromolecular Crystallography (CMC)] with the crystal structure of *C. pasteurianum* hydrogenase [10].

Results

Simulation model of Fe hydrogenase

Based on the X-ray structure of *C. pasteurianum* we modeled the iron hydrogenase of *C. acetobutylicum* (Fig. 1a). The simulation model of Fe-only hydrogenase closely fits to the X-ray crystal structure of the *C. pasteurianum* hydrogenase reported in literature [38]. The RMS deviation for the Cα atoms of the generated model from that of X-ray crystal structure of *C. pasteurianum* hydrogenase was 1.18 Å.

Immunoreactivity of antibody

The peptides of the hydrogenase sequence used for raising antibodies Hyd1, Hyd2, and Hyd3 are indicated in the model of hydrogenase (Fig. 1b). The negatively purified antibodies Hyd1 (1–16 amino acid residues), Hyd2 (415–428), and Hyd3 (424–441) raised against hydrogenase reacted with a 67-kDa protein in cell extracts of *C. acetobutylicum* (Fig. 2b–d). The antibodies produced a distinct signal that was absent in control using the preimmune sera (Fig. 2e). Western blot analysis of 67 kDa HydA recombinant protein (with removal of GST tag) expressed in *E. coli* demonstrated

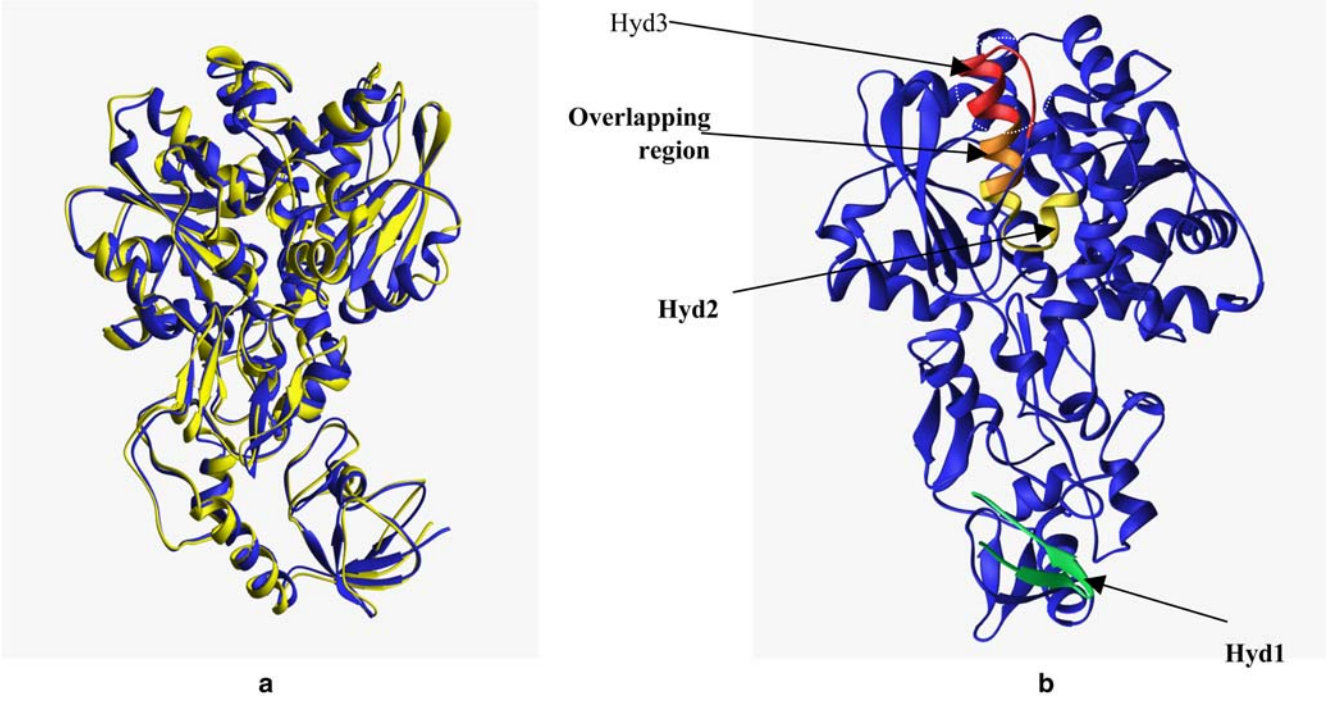


Fig. 1 **a** Simulation model of the 3D structure of the *Clostridium acetobutylicum* hydrogenase shown as ribbon (*white*) generated with the known 3D of the iron hydrogenase from *C. pasteurianum* by using the geno3D protein modeling software as described in "Materials and methods" section and superimposed by a Unix-based graphic program (Ribbons) with the crystal structure of *C. pasteurianum* hydrogenase (*black*). **b** Structure showing the peptides of the hydrogenase sequence used for raising antibodies. The *arrows* point to *light gray* Hyd1, *white* Hyd2, *encircled region* Hyd3, and *gray overlapping region* peptide common to Hyd2 and Hyd3

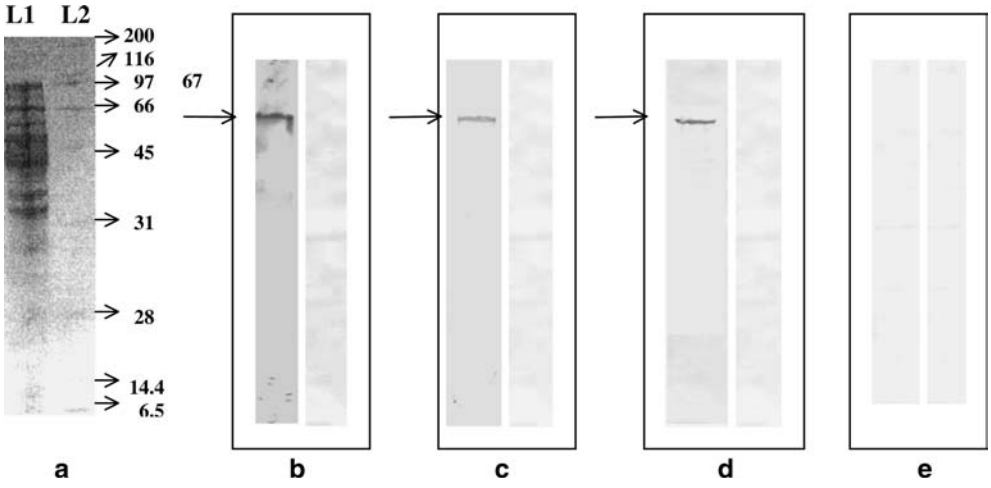


Fig. 2 Western blot analysis of antibodies. **a** SDS-PAGE of *C. acetobutylicum* cell extract stained with coomassie blue. *Lane 1* cell extract 10 μg; *Lane 2* molecular marker sizes in kDa; **b-e** Western blot probed with Hyd1, Hyd2, Hyd3 antibodies, and preimmune sera, respectively. The *arrow* points to the signal corresponding to the size of the hydrogenase band at 67 kDa

positive immunoreactivity with the Hyd1, Hyd2, and Hyd3 antibodies (Fig. 3).

In vitro site-specific inhibition studies of TNT transformation using designed antibodies

Western analysis of cell extracts using the antibodies purified by immunoadsorption demonstrated immunoreactivity towards 67-kDa protein in cell extracts. However, the signal obtained with Hyd2 antibody was weak. Hyd2 antibody in the range of 1:200–1:1,000 was analyzed for immunoreactivity. The transformation of TNT (100 μM) by crude cell extracts was completely inhibited by Hyd2 antibody (against the region of aminoacids 415–428) whereas antibodies Hyd1 (1–16 residues) and Hyd3 (424–448) inhibited 36 and 65%, respectively, at antibody titer of 1:10 whereas no inhibition was observed in presence of preimmune sera (Fig. 4a). The antibody titer of 1:5 also exhibited inhibition of 100, 65, and 36%, respectively, with Hyd2, Hyd3, and Hyd1 antibodies (data not shown). The inhibitory effect of different titers of Hyd2 antibody (1:10, 1:20, and 1:40) indicated a decrease in inhibitory effect on increasing the antibody dilution (Fig. 4b). Similar inhibition patterns were obtained with experi-

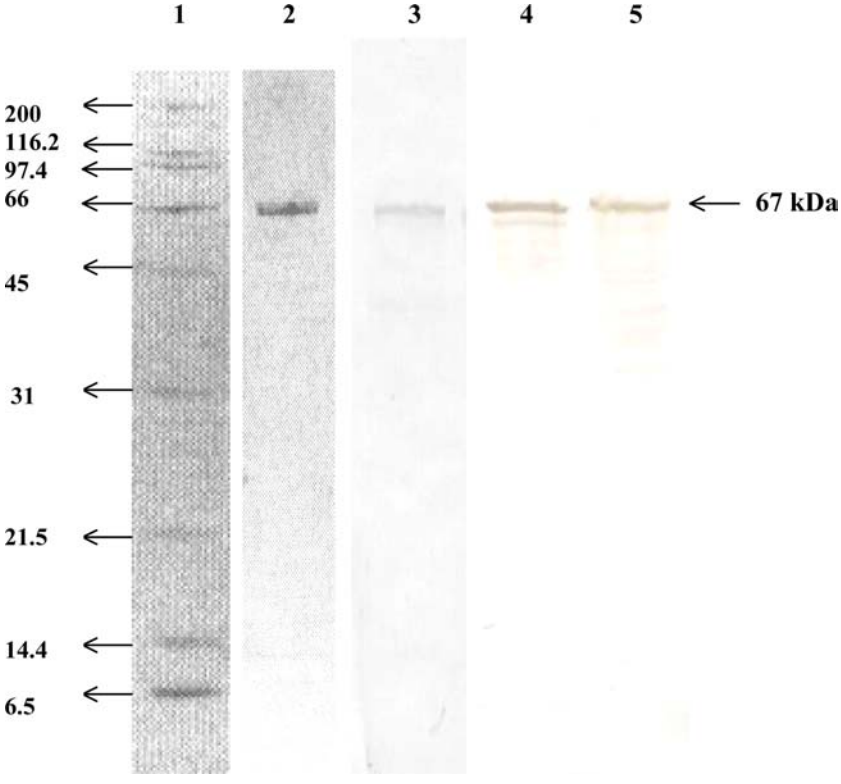
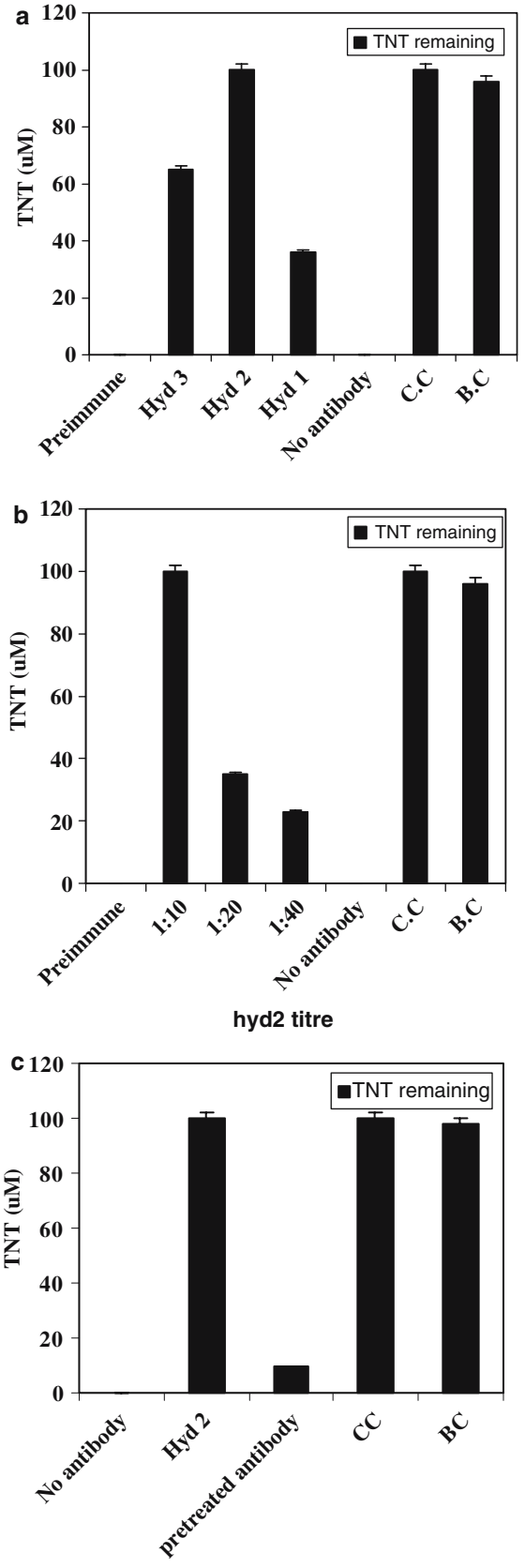


Fig. 3 Recombinant expressed HydA reacts with Hyd2 antibodies. HydA cloned in pET 41a vector was overexpressed in *E. coli* BL21 DE3 upon induction with IPTG. *Lane 1* SDS-PAGE marker of 200, 116.2, 97.4, 66, 45.0, 31.0, 21.5, 14.4, 6.5 kDa proteins; *Lane 2* purified recombinant HydA protein (with fusion tag removed) expressed in *E. coli* strain BL21 DE3 (5 μg); *Lane 3–5* Western blot probed with Hyd2, Hyd1, and Hyd3 antibody, respectively. The *arrow* points to the hydrogenase band at 67 kDa



◀

Fig. 4 Inhibitory effects of hydrogenase antibodies on TNT transformation. **a** Inhibitory effect of hydrogenase antibodies Hyd1, Hyd2, Hyd3 (1:10) on TNT reduction by *C. acetobutylicum* ATCC 824 cell extracts (100 µg protein) in 5 mM Tris buffer pH 7.8. In parallel, controls were maintained with no antibody, preimmune sera, chemical control *CC* (TNT in 5 mM Tris pH 7.8), and biological controls *BC* (the cell extract was first boiled and then incubated with TNT). The data presented is an average of triplicate experiments and the *error bars* are the standard deviations. **b** Inhibitory effect of different titers of Hyd2 (1:10, 1:20, and 1:40) on TNT reduction. Preimmune, chemical and biological controls are also shown as in **a**. **c** TNT transformation activity by *C. acetobutylicum* cell extracts (100 µg) carried out in presence of Hyd2 (1:10) antibodies pretreated with inactive recombinant hydrogenase (100 µg). *CC* chemical control (TNT in 5 mM Tris pH 7.8), *BC* biological control (cell extract was first boiled and then incubated with TNT)

ments performed with Hyd1 and Hyd3 antibodies (data not shown). The blocking of TNT transformation activity and the immunoreactivity of recombinant hydrogenase with the antibodies was further confirmed by using Hyd2 antibody pretreated with inactive recombinant hydrogenase in TNT transformation experiments by cell extracts. The pretreatment of the antibody with inactive hydrogenase removed the hydrogenase reacting antibody and 90% of the TNT transforming activity of the cell extract was retained (Fig. 4c).

Differential inhibition studies

Effect of procion red dye

The triazine dye, procion red, immobilized onto agarose has been used successfully as a ligand for the affinity chromatography purification of certain hydrogenases [28, 39, 40]. The inhibitory effect of procion red and other triazine dyes on several hydrogenases also has been demonstrated [19]. The inhibition of TNT reduc-

Table 1 Effect of procion red and metronidazole on TNT transformation by cell extracts of *Clostridium acetobutylicum*. The values are the averages of triplicate experiments (mean ± standard deviation)

	% Inhibition
Procion red (mM)	
0.0	0.0
0.25	52 ± 1
0.5	61 ± 5
1.0	80 ± 4
Metronidazole (mM)	
0.0	0
0.1	24 ± 2
0.2	35 ± 3
0.5	63 ± 1

A crude cell extract of *Clostridium acetobutylicum* containing 100 µg of protein was used. TNT (100 µM with procion red and 80 µM with metronidazole) reduction in 5 mM Tris pH 7.8 was monitored by HPLC

tion increased with an increase in the concentration of procion red (Table 1).

Effect of metronidazole

Trinitrotoluene reduction experiments by cell extracts of *C. acetobutylicum* in the presence of the nitroimidazole drug metronidazole were carried out to investigate if TNT transformation is inhibited in presence of metronidazole. TNT transformation was found to be inhibited in the presence of metronidazole (Table 1). At a concentration of 0.5 mM metronidazole 63% inhibition of TNT (80 μM) transformation was observed after 60 min incubation.

Effect of transition metal ions

The inhibitory effect of transition metals on TNT transformation using cell extracts of *C. acetobutylicum* indicates that Cu(2+) and Hg(2+) ions at 0.5 mM concentration could inhibit TNT transformation whereas the divalent cations of Ni and Co had no inhibitory effect. Growth of *C. acetobutylicum* was also inhibited at 0.5 mM of Cu(2+) and Hg(2+) ions whereas Co(2+) and Ni (2+) ions had no effect (Table 2).

Discussion

The aminoacid sequence of Fe-only hydrogenase of *C. acetobutylicum* shows 84% similarity to that of the Fe-only hydrogenase from *C. pasteurianum* hence could be inferred to be structurally identical. The deduced primary amino acid sequence alignments of known Fe-only hydrogenases show considerable identity and similarity indicating that they share a common structure [18, 31, 34].

There are rare reports concerning mechanistic aspects of TNT transformation by Clostridia. A preliminary catalytic mechanism for the carbon monoxide dehydrogenase (CODH) and Ni–Fe hydrogenase catalyzed reduction of TNT by *Clostridium thermoaceticum* was proposed [25]. In that study CODH from *C. thermoaceticum* was demonstrated to catalyze the reduction of TNT in the presence of CO and was inhibited by cyanide ion (a known inhibitor for the CO/CO_2 redox activity of the enzyme). The fact that cyanide inhibits the reaction and that adding CO restores activity indicates a CO activated site is required for TNT reduction and suggests that TNT may interact with the active site for $CO–CO_2$ redox catalysis, namely, the C cluster [4, 24].

The results of the inhibition studies of TNT transformation by copper and mercuric salts suggest that inhibition is a consequence of the destruction of the [4Fe–4S] centers in hydrogenase. Analogous investigations on the inhibitory effect of transition metals on the activity of *D. gigas* and *Desulfovibrio vulgaris* hydrogenase have been reported. Analysis of the EPR spectra of *D. vulgaris* hydrogenase treated by mercury salts suggests that iron and nickel atoms of the enzyme may be replaced by Hg(2+) [14, 27]. The irreversible inhibition of hydrogenase activity in purple sulfur bacterium *Thiocapsa roseopersicina* by Cu(2+) and Hg(2+) ions has been attributed to the degradation of Fe–S clusters by replacement of iron atoms of Fe–S clusters by copper or mercury ions [48]. Previous studies have indicated that procion red acts as a competitive inhibitor with respect to methyl viologen and noncompetitive with hydrogen in *D. gigas* hydrogenase [19]. The hydrogen evolution activity of *D. gigas* hydrogenase at 1 mM procion red concentration was inhibited by 90% whereas the tritium exchange activity which provides a direct measurement of activity at the site of hydrogen activation exhibited an inhibition of only 20%. This differential inhibition by the dye of the reactions observed involving electron carriers but not of hydrogen activation implies that procion red binds at a different site than that of hydrogen activation which is considered to take place at the H cluster (2). TNT (100 μM) reduction with *C. acetobutylicum* hydrogenase was 80% inhibited by procion red (1 mM) which corresponds to the degree of inhibition of hydrogen evolution activity in *D. gigas* hydrogenase. The identity of this site within the Fe-only hydrogenase of *C. acetobutylicum* remains to be established. The *D. gigas* hydrogenase is a Ni–Fe hydrogenase which differs from the Fe-only hydrogenase of *C. pasteurianum* in metal center at active site and in the number and type of iron sulfur clusters which probably serve as secondary electron carriers [9, 46]. It has been demonstrated that hydrogenase from *C. pasteurianum* reduces the nitroimidazole drug metronidazole, a strong electron acceptor via a ferredoxin linked mechanism. The above-mentioned study suggests the possible binding site for the drug metronidazole on hydrogenase as being in close proximation to the electron carrier site [11]. The inhibition of TNT reduction in the presence of metronidazole, a nitrosubstituent containing compound suggests that there are distinct binding domains for different substrates in Fe-only hydrogenase of *C. acetobutylicum*. The active site domain of Fe-only hydrogenase of *C. pasteurianum* contains amino acid residues 210–574 [38]. The Hyd2 and

Table 2 Effect of transition metals on TNT transformation by cell extracts of *Clostridium acetobutylicum*. The values are the averages of triplicate experiments (mean ± standard deviation)

Transition metal (0.5 mM)	% Inhibition	Growth (*Clostridium acetobutylicum*)
No transition metal	0.0	+
Cu	100 ± 1	–
Hg	100 ± 2	–
Ni	0.0	+
Co	0.0	+

A crude cell extract of *Clostridium acetobutylicum* containing 100 μg of protein was used. TNT reduction (100 μM) in 5 mM Tris pH 7.8 was monitored by HPLC
+ growth; – no growth

Hyd3 antibodies are against conserved peptides in this region of *C. acetobutylicum* Fe-only hydrogenase and hence could block TNT transformation activity. The relative lesser inhibition with the Hyd1 antibody which would react at an ionic surface further away from the active site and the observation of no inhibition in the presence of preimmune sera is consistent with this idea. The observation that antibody titers from 1:10 to 1:5 inhibited TNT transformation to the same degree suggested maximum antibody binding (complete saturation of all the available epitopes). The TNT transforming activity of the cell extract was retained when Hyd2 antibody pretreated with purified inactive recombinant hydrogenase was used indicating the specificity of binding the hydrogenase by the antibody preparation and limits concern about the nonspecific effect of the antibody. However, the limitation of this experiment is that the polyclonal antibodies could possibly bind with differing affinity to cause inhibition of enzyme activity and thus give less information on the portion of hydrogenase required for activity.

In conclusion the present study suggests that the TNT reducing activity of the *C. acetobutylicum* extracts under the conditions examined is essentially completely attributed to hydrogenase. Further studies are required to identify the specific site of TNT reduction on hydrogenase and to see if other enzymes encoded by *C. acetobutylicum* can contribute to TNT transformation under other growth or assay conditions.

Acknowledgement This material is based upon work supported by the US Army Research Office DOD ARMY W911NF-04-1-0179. We would like to thank Dr. M. S. Cates for assistance with the structure modeling.

References

1. Adams MWW (1990) The structure and mechanism of iron-hydrogenases. Biochim Biophys Acta 1020:115–145
2. Adams MWW, Mortenson LE, Chen JS (1981) Hydrogenases. Biochim Biophys Acta 594:105–176
3. Ahmad F, Hughes JB (2000) Anaerobic transformation of TNT by *Clostridium*. In: Spain JC, Hughes JB, Knackmuss HJ (eds) Biodegradation of nitroaromatic compounds and explosives. Lewis, Boca Raton, pp 185–212
4. Anderson ME, Lindahl PA (1994) Organization of clusters and internal electron pathways in CO dehydrogenase from *Clostridium thermoaceticum*: relevance to the mechanism of catalysis and cyanide inhibition. Biochemistry 33:8702–8711
5. Angermaier L, Hein F, Simon H (1981) Investigations on the reduction of aliphatic and aromatic nitro compounds by *Clostridium* species and enzyme systems. In: Bothe H, Trebst A (eds) Biology of inorganic nitrogen and sulphur. Springer, Berlin Heidelberg New York, pp 266–275
6. Benet C, Van Cutsem P (2002) Negative purification method for the selection of specific antibodies from polyclonal antisera. Biotechniques 33:1050–1054
7. Bruns-Nagel D, Scheffer S, Casper B, Garn H, Drzyzga O, Von Low E, Gemsa D (1999) Effect of 2,4,6-trinitrotoluene and its metabolites on human monocytes. Environ Sci Technol 33:2566–2570
8. Cammack R (1999) Hydrogenase sophistication. Nature 397:214–215
9. Cammack R, Fernandez VM, Hatchikian EC (1994) Nickel–iron hydrogenase. Methods Enzymol 243:43–68
10. Carson M (1987) Ribbon models of macromolecules. J Mol Graph 5:103–106
11. Church DL, Rabin HR, Laishley EJ (1988) Role of hydrogenase 1 of *Clostridium pasteurianum* in the reduction of metronidazole. Biochem Pharmacol 37:1525–1534
12. Combet C, Jambon M, Deleage G, Geourjon C (2002) Geno3D: automatic comparative molecular modeling of protein. Bioinformatics 18:213–214
13. Environmental Protection Agency. Online. (1993) Integrated risk information system data for TNT, vol 2002. http://www.epa.gov/iris/subst/0269.htm
14. Fernandez VM, Rua ML, Reyes P, Cammack R, Hatchikian EC (1989) Inhibition of *Desulfovibrio gigas* hydrogenase with copper salts and other metal ions. Eur J Biochem 185:449–454
15. Florin L, Tsokoglou A, Happe T (2001) A novel type of iron hydrogenase in the green alga *Scenedesmus obliquus* is linked to the photosynthetic electron transport chain. J Biol Chem 276:6125–6132
16. Gaillard J, Quinkal I, Moulis JM (1993) Effect of replacing conserved proline residues on the EPR and NMR properties of *Clostridium pasteurianum* 2[4Fe–4S] ferredoxin. Biochemistry 32:9881–9887
17. Garcia-Sanchez MI, Diaz-Quintana A, Gotor C, Jacquot JP, De la Rosa MA, Vega JM (2000) Homology predicted structure and functional interaction of ferredoxin from the eukaryotic alga *Chlamydomonas reinhardtii* with nitrite reductase and glutamate synthase. J Biol Inorg Chem 5:713–719
18. Gorwa MF, Croux C, Soucaille P (1996) Molecular characterization and transcriptional analysis of the putative hydrogenase gene of *Clostridium acetobutylicum* ATCC824. J Bacteriol 178:2668–2675
19. Hallahan DL, Fernandez VM, Hatchikian EC, Hall DO (1986) Differential inhibition of catalytic sites in *Desulfovibrio gigas* hydrogenase. Biochimie 68:49–54
20. Hallas LE, Alexander M (1983) Microbial transformation of nitroaromatic compounds in sewage effluent. Appl Environ Microbiol 45:1234–1241
21. Hartmanis MGN, Gatenbeck S (1984) Intermediary metabolism in *Clostridium acetobutylicum*: levels of enzymes involved in the formation of acetate and butyrate. Appl Environ Microbiol 47:1277–1283
22. Honeycutt ME, Jarvis AS, McFarland VA (1996) Cytotoxicity and mutagenicity of 2,4,6-trinitrotoluene and its metabolites. Ecotoxicol Environ Saf 35:282–287
23. Horner DS, Heil B, Happe T, Embley TM (2002) Iron hydrogenases—ancient enzymes in modern eukaryotes. Trends Biochem Sci 27:148–153
24. Hu X, Spangler NJ, Anderson ME, Xia J, Ludden PW, Lindahl PA, Münck E (1996) Nature of the C-cluster in Ni-containing carbon monoxide dehydrogenase. J Am Chem Soc 118:830–845
25. Huang S, Lindahl PA, Wang C, Bennett GN, Rudolph FB, Hughes JB (2000) 2,4,6-Trinitrotoluene reduction by carbon monoxide dehydrogenase from *Clostridium thermoaceticum*. Appl Environ Microbiol 66:1474–1478
26. Hughes JB, Wang CY, Yesland K, Richardson A, Bhadra R, Bennett GN, Rudolph FB (1998) Bamberger rearrangement during TNT metabolism by *Clostridium acetobutylicum*. Environ Sci Technol 32:494–500
27. Kamachi T, Seiichi U, Hirashi T, Okura I (1995) Effect of Hg(II) on hydrogenase activity from *Desulfovibrio vulgaris* (Miyazaki). J Mol Catal A—Chem 96:329–333
28. Knuttel K, Schneider K, Schlegel HG, Muller A (1989) The membrane-bound hydrogenase from *Paracoccus denitrificans*. Purification and molecular characterization. Eur J Biochem 179:101–108
29. Kummerle R, Kyritsis P, Gaillard J, Moulis JM (2000) Electron transfer properties of iron–sulfur proteins. J Inorg Biochem 79:83–91

30. Lockerby DL, Rabin HR, Laishley EJ (1985) Role of the phosphoroclastic reaction of *Clostridium pasteurianum* in the reduction of metronidazole. Antimicrob Agents Chemother 27:863–867
31. Meyer J, Gagnon J (1991) Primary structure of hydrogenase I from *Clostridium pasteurianum*. Biochemistry 30:9697–9704
32. Montet Y, Amara P, Volbeda A, Vernede X, Hatchikian EC, Field MJ, Frey M, Fontecilla-Camps JC (1997) Gas access to the active site of Ni–Fe hydrogenases probed by X-ray crystallography and molecular dynamics. Nat Struct Biol 4:523–526
33. Moulis JM, Davasse V (1995) Probing the role of electrostatic forces in the interaction of *Clostridium pasteurianum* ferredoxin with its redox partners. Biochemistry 34:16781–16788
34. Nicolet Y, Piras C, Legrand P, Hatchikian CE, Fontecilla-Camps JC (1999) *Desulfovibrio desulfuricans* iron hydrogenase: the structure shows unusual coordination to an active site Fe binuclear center. Structure 7:13–23
35. Nicolet Y, Lemon BJ, Fontecilla-Camps JC, Peters JW (2000) A novel FeS cluster in Fe-only hydrogenases. Trends Biochem Sci 25:138–143
36. Noyes R (1996) Chemical weapons destruction and explosive waste/unexploded ordinance remediation. Noyes, New Jersey, pp 102–141
37. Peters JW (1999) Structure and mechanism of iron-only hydrogenases. Curr Opin Struct Biol 9:670–676
38. Peters JW, Lanzilotta WN, Lemon BJ, Seefeldt LC (1998) X-ray crystal structure of the Fe-only hydrogenase (CpI) from *Clostridium pasteurianum* to 1.8 angstrom resolution. Science 282:1853–1858
39. Pinkwart M, Schneider K, Schlegel HG (1983) Purification and properties of the membrane-bound hydrogenase from N2-fixing *Alcaligenes latus*. Biochim Biophys Acta 745:267–278
40. Podzuweit HG, Schneider K, Knuttel H (1987) Comparison of the membrane-bound hydrogenases from *Alcaligenes eutrophus* H16 and *Alcaligenes eutrophus* type strain. Biochim Biophys Acta 905:435–446
41. Preuss A, Fimpel J, Diekert G (1993) Anaerobic transformation of 2,4,6-trinitrotoluene (TNT). Arch Microbiol 159:345–353
42. Quinkal I, Davasse V, Gaillard J, Moulis JM (1994) On the role of conserved proline residues in the structure and function of *Clostridium pasteurianum* 2[4Fe–4S] ferredoxin. Protein Eng 7:681–687
43. Rieger PG, Knackmuss HJ (1995) Basic knowledge and perspectives on biodegradation of 2,4,6-trinitrotoluene and related nitroaromatic compounds in contaminated soil. In: Spain JC (ed) Biodegradation of nitroaromatic compounds, vol 49. Plenum Press, New York, pp 1–18
44. Sani RK, Peyton BM, Brown LT (2001) Copper-induced inhibition of growth of *Desulfovibrio desulfuricans* G20: assessment of its toxicity and correlation with those of zinc and lead. Appl Environ Microbiol 67:4765–4772
45. Schneider K, Pinkwart M, Jochim K (1983) Purification of hydrogenases by affinity chromatography on Procion Red-agarose. Biochem J 213:391–398
46. Volbeda A, Charon MH, Piras C, Hatchikian EC, Frey M, Fontecilla-Camps JC (1995) Crystal structure of the nickel–iron hydrogenase from *Desulfovibrio gigas*. Nature 373:580–587
47. Watrous MW, Clark S, Kutty R, Huang S, Rudolph FB, Hughes JB, Bennett GN (2003) 2,4,6-Trinitrotoluene reduction by an Fe-only hydrogenase in *Clostridium acetobutylicum*. Appl Environ Microbiol 69:1542–1547
48. Zadvorny OA, Zorin NA, Gogotov IN (2000) Influence of metal ions on hydrogenase from purple sulfur bacterium *Thiocapsa roseopersicina*. Biochemistry 65:1287–1291