Purification of the nickel protein carbon monoxide dehydrogenase of *Clostridium thermoaceticum*

Gabriele Diekert and Maria Ritter

Fachbereich Biologie/Mikrobiologie, Philipps-Universität, Lahnberge, D-3550 Marburg/Lahn, FRG

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The carbon monoxide dehydrogenase was purified from *Clostridium thermoaceticum* to apparent homogeneity. The 120-fold purified enzyme with app. M_r 250000 had a nickel content of 10 ± 2 μmol Ni/protein.

Carbon monoxide dehydrogenase *Acetogenic bacteria* Clostridium thermoaceticum *Nickel enzyme*

1. INTRODUCTION

The synthesis of carbon monoxide dehydrogenase of several anaerobic bacteria was shown to be dependent on nickel [1–3]. With the partially purified enzyme (14-fold) of *Clostridium thermoaceticum* co-chromatography of ^{63}Ni and CO dehydrogenase activity was observed on polyacrylamide gels [4]. Here the purification of CO dehydrogenase from *C. thermoaceticum* to apparent homogeneity is described. The nickel content of the 120-fold purified enzyme (34% yield) was determined to be 10 ± 2 μmol/g protein. Gradient gel electrophoresis revealed an app. M_r of the native enzyme of near 250000, which corresponds to a nickel content of 2–3 mol Ni/mol enzyme. During the last purification steps the turnover number/Ni remained constant. These findings conclusively demonstrate that CO dehydrogenase is a nickel protein.

2. MATERIALS AND METHODS

2.1. *Growth of* Clostridium thermoaceticum

C. Thermoaceticum (DSM 521) was grown at 55°C in an anaerobic glucose medium [5] containing 1.5 μM $^{63}NiCl_2$ (50 μCi/μmol). The cells were harvested by centrifugation for 30 min at $16000 \times g$ under a N_2 atmosphere, and washed twice with buffer I (50 mmol Tris–HCl (pH 8.0) plus 3.91 g 2-mercaptoethanol, 1.1 g sodium pyruvate, 8.7 mg phenylmethylsulfonylfluoride, and 25.7 mg methyl viologen/l [4]). This buffer was previously gassed with carbon monoxide, and then ~1–5 μM sodium dithionite were added to yield a slight blue colour of the reduced methyl viologen, which indicated the absence of oxygen.

2.2. *Determination of enzymic activity and of protein*

Carbon monoxide dehydrogenase activity was determined as in [5] using methyl viologen as artificial electron acceptor. One unit of activity corresponds to 1 μmol CO oxidized/min at 55°C and pH 7.0. Protein was measured by the biuret method with KCN [6].

2.3. *Determination of nickel*

^{63}Nickel was determined by counting the radioactivity. The specific radioactivity of ^{63}Ni was calculated by measuring the Ni content and the radioactivity of the 120-fold purified enzyme. The Ni determination was performed by Dr G. Wolff (Zentrallabor für Chemische Analysen, Kernforschungsanlage, Jülich) using emission spectroscopy with inductively-coupled plasma.

2.4. *Purification of carbon monoxide dehydrogenase*

All purification steps were performed anaerobi-

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cally under carbon monoxide as gas phase at room temperature. ^{63}Ni-Labelled cells (5 g wet wt) corresponding to ~500 mg protein were suspended in 10 ml buffer I (section 2.1) containing 10 mg deoxyribonuclease I (Boehringer, Mannheim). Cell extracts were prepared by passing the suspension twice through a french pressure cell at 137000 kPa (20000 lb/in.^{2}). Cell debris was removed by centrifugation for 30 min at 30000 × *g*. The supernatant (cell extract) was applied to a DEAE–Sepharose CL-6B column (1 cm diam., 12 cm length) pre-equilibrated with buffer I. The column was then washed with 100 and 200 mM NaCl in buffer I, each 20 ml, and the CO dehydrogenase was eluted with 300 mM NaCl. The eluate was collected in pre-weighed 10-ml serum-bottles containing 0.2 ml 0.16% (w/v) dithioerythritol, 5 μl 100 mM methyl viologen and carbon monoxide as far phase. The fraction volume was 1–5 ml and was determined by weighing the serum-bottles. Elution of active carbon monoxide dehydrogenase was indicated by an immediate change of colour in the collected fraction due to the reduction of methyl viologen. To the pooled fractions containing CO dehydrogenase (total volume ~5 ml) a 100% saturated ammonium sulfate solution in buffer I was added up to 45% final conc. The suspension was then centrifuged for 15 min at 30000 × *g*. The supernatant, which contained 70–90% of the carbon monoxide dehydrogenase, was applied to a further DEAE–Sepharose column (1 cm × 6 cm), pre-equilibrated with 40% saturated ammonium sulfate in buffer I. The column was washed with 10 ml 30% saturated ammonium sulfate, and the enzyme was eluted with 15% ammonium sulfate (total volume of the active fractions ~3 ml).

The pooled active enzyme was then passed through a phenyl–Sepharose column (1 cm × 5 cm), pre-equilibrated with 20% saturated ammonium sulfate in buffer I. After washing with 10% ammonium sulfate (10 ml) the enzyme was eluted with buffer I (total volume of the CO dehydrogenase containing fractions of ~2 ml).

2.5. *Gel electrophoresis*

Polyacrylamide gel electrophoresis was performed in 7.5% polyacrylamide gels as in [7].

Gradiant gel electrophoresis for the determination of the app. M_r of the native enzyme was run in a 5–30% polyacrylamide gel gradient [8]. Linearity of the gradient was examined photometrically by scanning the gels at 578 nm after preparation of the gels using bromphenolblue in the 5% polyacrylamide solution. Only those gels showing a linear gradient from top to bottom were used. The migration distance of active CO dehydrogenase was determined by activity-staining of the gel under CO gas phase in the presence of methyl viologen [4].

SDS-gel electrophoresis was performed in 10% SDS–polyacrylamide gels as in [9]. The M_r of the CO dehydrogenase subunits was calculated from their relative mobility compared to that of marker proteins with a known M_r. All gels were stained with Coomassie blue G-250.

3. RESULTS

3.1. *Purification of CO dehydrogenase*

Due to the extreme oxygen sensitivity of the enzyme all purification steps were performed under strict anaerobic conditions in the presence of CO and methyl viologen. The purification procedure is summarized in table 1. It should be noted that from the DEAE–Sepharose I, two peaks of enzyme activity were eluted, one at 200 mM NaCl and the other at 300 mM NaCl. The first peak contained only ~10% of the total activity applied to the column and was discarded. The enzyme obtained in the second peak was purified to apparent homogeneity by ammonium sulfate fractionation and chromatography on DEAE–Sepharose and phenyl–Sepharose. The 120-fold purified CO dehydrogenase migrated as a single protein band in 7.5% polyacrylamide gels.

Table 1

Purification of carbon monoxide dehydrogenase from *Clostridium thermoaceticum*

Purification state	Spec. act. (Units/mg)	Yield (%)	Nickel (nmol/mg)
Cell extract	3.6	100	2.6
DEAE-Sepharose I	46.7	92	3.2
Ammonium sulfate fractionation	85.9	81	2.4
DEAE-Sepharose II	176.7	56	4.8
Phenyl-Sepharose	435.0	34	10.0

3.2. *Ni content*

During the purification of the enzyme the increase in nickel content/mg protein paralleled the increase in specific enzyme activity only from the ammonium sulfate precipitation onwards. This indicates that some Ni-containing material other than CO dehydrogenase was removed by the first steps of purification. The Ni content of the purified enzyme was found to be 10 μmol/g protein but it varied within a limited range (± 2 μmol/g) in different preparations. From this value, and the specific activity of the enzyme, a turnover number of ~700 s^{-1}/Ni can be estimated.

3.3. *App. M_r of the native enzyme and of the subunits*

Gradient gel electrophoresis of the enzyme revealed an app. M_r of the native enzyme of near 250000 (fig. 1). Based on this M_r-value a nickel content of 2–3 mol/mol enzyme can be calculated. In [4] a M_r of 410000, determined by gel filtration of the 14-fold enzyme was reported.

SDS-gel electrophoresis showed two major protein bands, the app. M_r of which were determined to be about 84000 or 72000, respectively (fig. 2). A weak band with a M_r of 12000 was also observed. It is not known whether this peptide belongs to the CO dehydrogenase. The exact molar ratio of the 3 peptides was not determined.

The gradient gel electrophoresis, as well as the SDS gel electrophoresis, was carried out im-

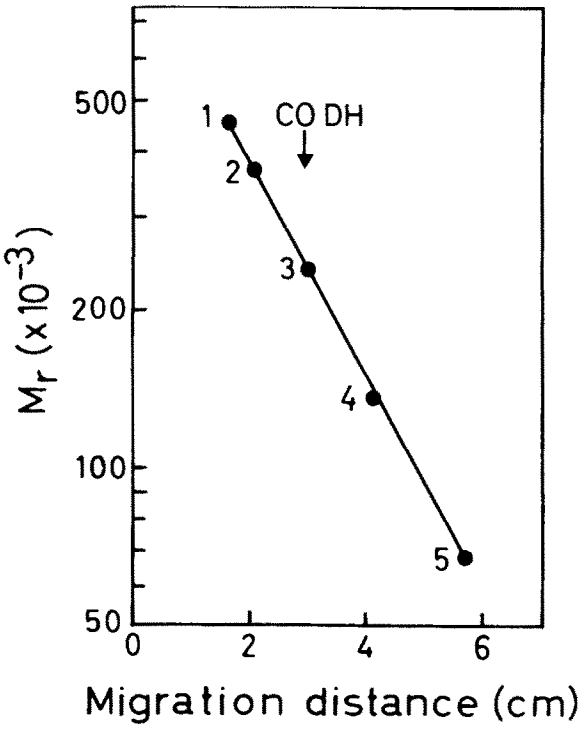

Fig. 1. Determination of the app. M_r of the native carbon monoxide dehydrogenase by gradient gel electrophoresis. The M_r markers were ($\times 10^{-3}$): 1, ferritin (455); 2, phosphorylase A (370); 3, catalase (240); 4, bovine serum albumin dimer (136); 5, bovine serum albumin monomer (68); (→) migration distance of CO dehydrogenase activity.

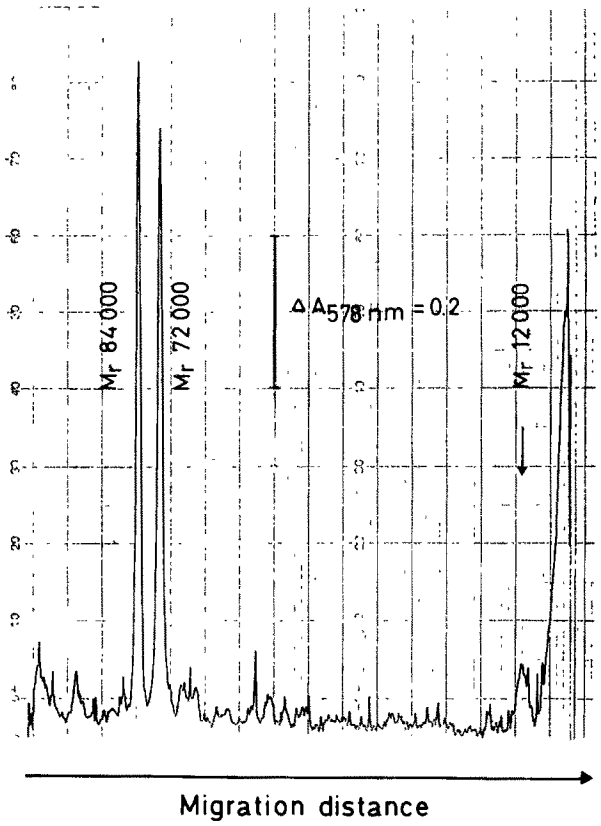

Fig. 2. SDS gel electrophoresis of the CO dehydrogenase of *C. thermoaceticum*. The electrophoresis was carried out with 10 μg of the enzyme preparation obtained from the expt of table 1. The gel was stained with Coomassie blue G-250 and scanned photometrically at 578 nm. M_r markers were ($\times 10^{-3}$): phosphorylase A (subunits, 92.5); bovine serum albumin (68); egg albumin (43); chymotrypsinogen (25.7); cytochrome *c* (11.7).

mediately after elution of the purified enzyme from the phenyl–Sepharose. This was necessary because of the lability of the purified enzyme. Within a few hours (accelerated by freezing the enzyme) the purified CO dehydrogenase spontaneously precipitated, accompanied by a complete loss of activity.

4. CONCLUSION

The purified CO dehydrogenase from *C. thermoaceticum* contained 2–3 mol Ni/mol. During the last purification steps the turnover no./Ni remained essentially constant. These findings and our observation that synthesis of CO dehydrogenase in Clostridia is dependent on nickel [1–3], conclusively demonstrate that CO dehydrogenase of *C. thermoaceticum* is a nickel protein.

While we were finishing this manuscript, EPR studies on a 40-fold purified CO dehydrogenase of *C. thermoaceticum* were published [10].

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