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Characterization of cinnamyl alcohol dehydrogenase of *Helicobacter pylori*

An aldehyde dismutating enzyme

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Cinnamyl alcohol dehydrogenases (CAD; 1.1.1.195) catalyse the reversible conversion of *p*-hydroxycinnamaldehydes to their corresponding alcohols, leading to the biosynthesis of lignin in plants. Outside of plants their role is less defined. The gene for cinnamyl alcohol dehydrogenase from *Helicobacter pylori* (HpCAD) was cloned in *Escherichia coli* and the recombinant enzyme characterized for substrate specificity. The enzyme is a monomer of 42.5 kDa found predominantly in the cytosol of the bacterium. It is specific for NADP(H) as cofactor and has a broad substrate specificity for alcohol and aldehyde substrates. Its substrate specificity is similar to the well-characterized plant enzymes. High substrate inhibition was observed and a mechanism of competitive inhibition proposed. The enzyme was found to be capable of catalysing the dismutation of benzaldehyde to benzyl alcohol and benzoic acid. This dismutation reaction has not been shown previously for this class of alcohol dehydrogenase and provides the bacterium with a means of reducing aldehyde concentration within the cell.

Cinnamyl alcohol dehydrogenases (CAD; EC 1.1.1.195) are zinc dependent dehydrogenases and are among the least studied of the alcohol dehydrogenase enzymes. The function of CADs in plants has been well characterized, where they have been shown to catalyse the reversible conversion of *p*-hydroxycinnamaldehydes to their corresponding alcohols leading to lignin biosynthesis [1–7].

Outside of plants the role of CAD is less well understood and the enzyme has only been kinetically characterized in two other species, *Mycobacterium bovis* BCG and *Saccharomyces cerevisiae* [8–10]. A role for CAD in lipid metabolism within the cell envelope was proposed in *M. bovis* BCG [8]. In *S. cerevisiae* it has been suggested that CAD may be involved in the Erlich pathway, the process whereby amino acids are degraded, leading to the formation of aldehydes which are subsequently metabolized via the activity of alcohol dehydrogenases (ADHs) to form fusel alcohols [9,10]. Although the CADs of *M. bovis* BCG and *S. cerevisiae* are not involved in lignin biosynthesis, they have similar substrate specificities to plant CADs.

The annotated genome of *H. pylori* strain 26695 [11] identifies a single putative *CAD* gene (*HP1104*) that we have cloned and characterized in an effort to gain a better understanding of this class of CAD outside of plants. The *H. pylori* CAD (HpCAD) was also of interest as its production was shown to increase 24-fold under acid stress conditions [12] and antibodies to HpCAD have been identified in the sera of gastric cancer patients [13].

Abbreviations
ADH, alcohol dehydrogenase; HpCAD, *Helicobacter pylori* cinnamyl alcohol dehydrogenase.

H. pylori is implicated in the pathogenesis of chronic gastritis and, more recently, in the development of gastric carcinoma [14–17]. The mechanisms whereby this organism causes damage to the gastric mucosa are not fully understood. However, strains possessing the vacuolating toxin (VacA) and the cytotoxin-associated antigen (CagA), which is used as a marker for the insertion of a pathogenicity island (*cag* PAI), are associated with a higher frequency of duodenal ulcer, atrophic gastritis and gastric carcinoma among infected patients [18]. In addition, other researchers have proposed that ADHs contribute to the pathogenicity of *H. pylori* by metabolizing dietary alcohols to form toxic aldehydes, which interact with the gastric mucosa to cause inflammation [19–27]. This paper reports the genetic cloning, production and characterization of HpCAD and the first demonstration that a member of the CAD family has an aldehyde dismutase activity.

Results

Overproduction of the *H. pylori* cinnamyl alcohol dehydrogenase

The putative *CAD* gene (*HP1104*) was clearly present in the strains of *H. pylori* tested (1061, 26695 and G27) (Fig. 1A). The corresponding protein product was detected by Western blotting in the above strains as well as in strain N6 (Fig. 1B). Genomic DNA from the sequenced strain 26695 was used for subsequent cloning studies. The *CAD* gene was cloned in *Escherichia coli* DH5α and the pET-Hp1104 construct containing the cloned gene was transformed into *E. coli* BL21(DE3)plysS for overexpression. A 600 mL preparation of pET-Hp1104 transformed *E. coli* BL21(DE3)plysS typically yielded approximately 12–18 mg of purified CAD. The His-tag on the N terminus of the expressed HpCAD protein facilitated a one-step affinity purification on a nickel-charged iminodiacetic acid column. The pure fractions of HpCAD eluted from the column were combined and dialysed against 75 mM sodium phosphate (pH 7.5) containing 5 mM dithiothreitol. The enzyme was stored in this buffer at −20 °C and no loss of activity was observed over 1 month. The presence of dithiothreitol in the buffer was required to prevent precipitation of the protein during dialysis.

SDS/PAGE analysis of the purified CAD by Coomassie Blue staining revealed a single band at 42.5 kDa (Fig. 2A) and the molecular mass from size exclusion chromatography was estimated to be 50 kDa (Fig. 2B). An absence of dithiothreitol from the buffer during gel filtration chromatography resulted in HpCAD forming higher molecular mass aggregates, presumably due to oxidation of the multiple cysteine residues present in HpCAD.

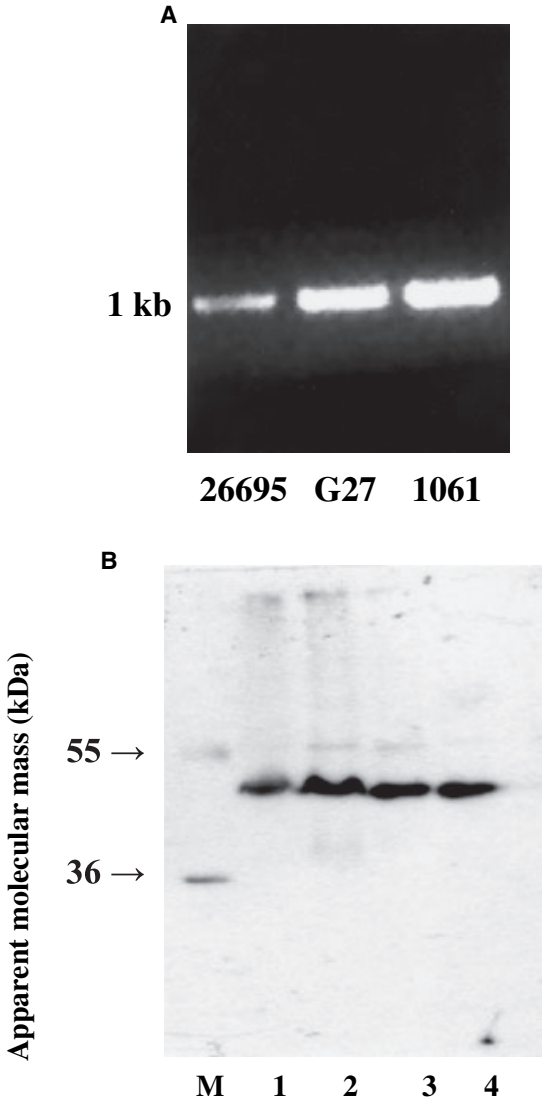


Fig. 1. PCR amplification of HpCAD in different *H. pylori* strains. (A) The PCR amplification of the *cinnamyl alcohol dehydrogenase* gene from strains 1061, 26695 and G27. The primers used were designed using strain 26695 as the template (http://www.Tigr.org). (B) An affinity purified polyclonal antibody raised in rabbits against HpCAD (1 : 200), was used to probe the cytosolic fractions of *H. pylori* strains 26695, 1061, G27 and N6. Protein (50 μg) is present in each lane. The blot was developed by enhanced chemiluminescence: lane 1, strain 26695; lane 2, strain 1061; lane 3, strain G27 and lane 4, strain N6.

Subcellular localization

A previous study of CAD from *M. bovis* BCG showed that 10–20% of the enzyme was associated with the

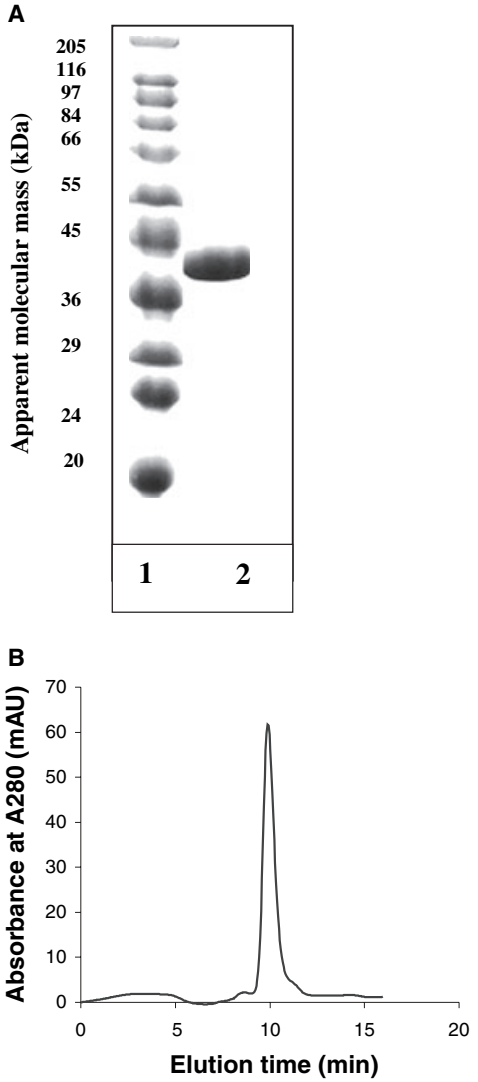


Fig. 2. (A) SDS/PAGE and gel filtration analysis of HpCAD. A sample of HpCAD (lane 2) eluted from the nickel charged iminodiacetic acid column was subjected to SDS/PAGE (15% acrylamide). The gel was stained with Coomassie Blue revealing a single band at 42.5 kDa. The molecular mass markers are shown in lane 1. (B) The profile of HpCAD (0.2 mg) after gel filtration over Superdex 75-HR, a single peak eluting a 9.89 min was observed.

cell envelope of this organism and a role for CAD in lipid metabolism within the envelope was postulated [8]. We examined the subcellular localization of HpCAD in *H. pylori* (Fig. 3), using an affinity-purified antibody against recombinant HpCAD. The majority of the immunoreactive material was found in the cytoplasmic fraction (Fig. 3; lane 2). Detectable amounts of immunoreactivity were also observed in the total envelope fraction (Fig. 3; lane 3). However, this may represent contamination of the envelope fraction with cytosolic components as no immunoreactivity was

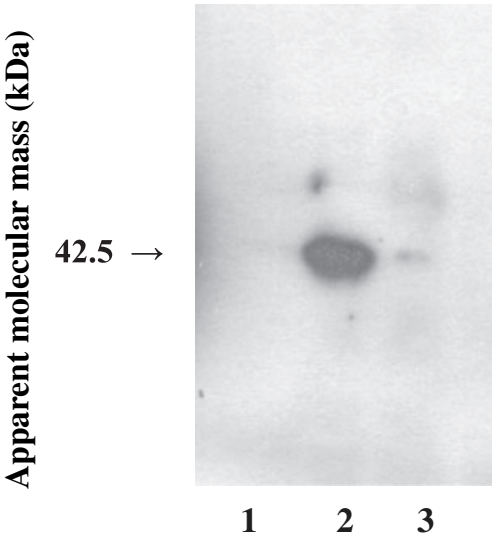


Fig. 3. Subcellular localization of HpCAD. Subcellular fractions of *H. pylori* were analysed by SDS/PAGE, transferred to poly(vinylidene difluoride) membrane and probed using an affinity purified polyclonal antibody against HpCAD (1 : 200). The blot was developed by ECL with a peroxidase conjugated anti-(rabbit IgG) Ig (1 : 1000), lane 1; inner membrane fraction, lane 2; cytosolic fraction and lane 3; total envelope fraction. Approximately 10 μg of protein was loaded in lanes 1–3.

observed in either the inner (Fig. 3; lane 1) or the outer membrane fractions (not shown).

CAD substrate specificity, kinetic parameters and sequence analyses

The *HP1140* gene product of *H. pylori* 26695 is active as a cinnamyl alcohol dehydrogenase. The substrate specificity of the pure enzyme was analysed for several aromatic and aliphatic substrates. The values of the steady-state parameters are summarized in Table 1. The best alcohol substrate was cinnamyl alcohol with a k_{cat}/K_m value of 126 $s^{-1}\cdot mM^{-1}$. Aliphatic alcohols were poorer substrates, with k_{cat}/K_m values 10-fold or more lower than the aromatic alcohols. The k_{cat}/K_m values for aldehydes were higher than those for alcohols. Of the aldehydes, cinnamaldehyde was the best substrate. Acetaldehyde had a 10-fold lower k_{cat}/K_m value than cinnamaldehyde. Given these substrate specificities we can confirm the *HP1104* gene product is a cinnamyl alcohol dehydrogenase, a putative function that was assigned by TIGR based on homology studies. In general, the substrate specificity was quite similar to that of the *S. cerevisiae*, *M. bovis* BCG and plant cinnamyl alcohol dehydrogenases. NADP(H) was the preferred coenzyme and the enzyme showed no activity with NAD^+ (up to a concentration of 2 mM).

Table 1. Kinetic parameters of *H. pylori* cinnamyl alcohol dehydrogenase. Enzymatic activities were measured in 75 mM sodium phosphate buffer (pH 7.5) with 2 mM $NADP^+$ for oxidation and 0.5 mM NADPH for reduction. All parameters were determined at 37 °C.

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)
Cinnamyl alcohol	0.10 ± 0.04	13.3 ± 1.7	126 ± 55
Coniferyl alcohol	0.11 ± 0.07	3.5 ± 1.3	32 ± 23
Benzyl alcohol	0.41 ± 0.05	8.4 ± 0.5	21 ± 3
Ethanol	46 ± 1	7.1 ± 0.6	0.15 ± 0.01
Propanol	13 ± 2	12.81 ± 0.8	0.96 ± 0.16
Butanol	9 ± 2	5.7 ± 0.2	0.63 ± 0.14
NADP[a]	0.06 ± 0.01	7.7 ± 0.6	128 ± 24
Cinnamaldehyde	0.005 ± 0.0001	27.4 ± 1.3	5480 ± 285
Coniferylaldehyde	0.008 ± 0.0002	2.3 ± 1	288 ± 125
Benzaldehyde	0.03 ± 0.002	16.71 ± 3.3	557 ± 116
Acetaldehyde	0.04 ± 0.002	25.2 ± 2.9	630 ± 79
NADPH[b]	0.15 ± 0.03	15.5 ± 1.8	103 ± 6
Dismutation	≈ 31	≈ 2.5	≈ 0.08

[a] Determined with benzyl alcohol at 5 mM. [b] Determined with acetaldehyde at 8 mM.

The highest catalytic activities were observed for the reduction of aldehyde substrates.

An alignment of HpCAD (strain 26695) with CADs from *H. pylori* strain J99, *Campylobacter jejuni*, *M. bovis* BCG, *S. cerevisiae* and *Eucalyptus gunnii* demonstrated that the regions of strongest sequence identity occurred in CADs from other bacterial organisms, i.e. *H. pylori* J99, *C. jejuni* and *M. bovis* BCG (96%, 63% and 42%, respectively). The CADs from the more distantly related *S. cerevisiae* (30%, 27%) and *E. gunnii* (14%) had fewer conserved regions, based on this sequence identity analysis.

High substrate inhibition

HpCAD activity was inhibited by high alcohol and aldehyde substrate concentrations. The degree of high substrate inhibition occurring during alcohol oxidation was related to the structure of the alcohol substrate employed. The aliphatic alcohol substrates, propanol and butanol, produced an inhibition which was less pronounced than that observed for the aromatic alcohol substrates, cinnamyl alcohol, coniferyl alcohol and benzyl alcohol. The initial rates of $NADP^+$ reduction were determined at a series of $NADP^+$ concentrations in the presence of fixed propanol concentrations at which high-substrate inhibition was apparent (100 mM and above). The results, presented as double-reciprocal plots for illustrative purposes (Fig. 4), indicate that the family of lines do not intersect at a common point. This would be consistent with a competitive mechanism in which high concentrations of the alcohol substrate exclude the binding of $NADP^+$, as depicted in the mechanism outlined below:

$$\begin{array}{ccccc} E & \rightleftharpoons & E.NADP^+ & \rightleftharpoons & E.NADP^+.Alc \\ \downarrow\uparrow & & & & \downarrow\uparrow \\ E.Alc & & & & E.NADPH.Ald \\ & & & & \downarrow\uparrow \\ & & & & E.NADPH \\ & & & & \downarrow\uparrow \\ & & & & E \end{array}$$

(where Alc and Ald represent the alcohol substrate and aldehyde product, respectively). This mechanism will give an initial-rate equation (Eqn 1) of the form [28,29]:

$$v = \frac{V_{max}}{1 + \frac{K_m^{NAD}}{[NAD^+]}\left(1 + \frac{[Alc]}{K_i}\right) + \frac{K_m^{Alc}}{[Alc]} + \frac{K_s^{NAD} K_m^{Alc}}{[NAD^+][Alc]}\left(1 + \frac{[Alc]}{K_i}\right)} \quad (1)$$

This indicates that the slopes of the lines (apparent K_m/V_{max} values) shown in Fig. 4 will not be a linear function of the propanol concentration. Similar behaviour would be expected for this type of substrate inhibition were the enzyme to follow other kinetic mechanisms, such as the Theorell–Chance mechanism or random-order mechanism under conditions

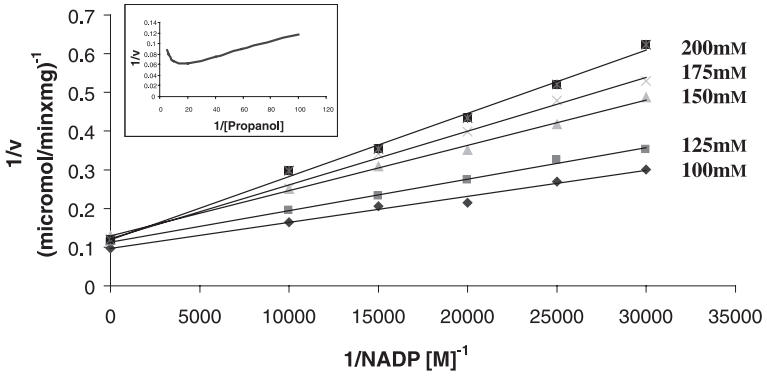


Fig. 4. High substrate inhibition of HpCAD by propanol. A double-reciprocal plot for varying concentrations of propanol at a fixed concentration of $NADP^+$ coenzyme shows substrate inhibition occurring at concentrations above 50 mM propanol (inset). The type of inhibition by propanol was examined using inhibitory concentrations of propanol and varying $NADP^+$ concentrations. From the data presented, the inhibition appears to be competitive.

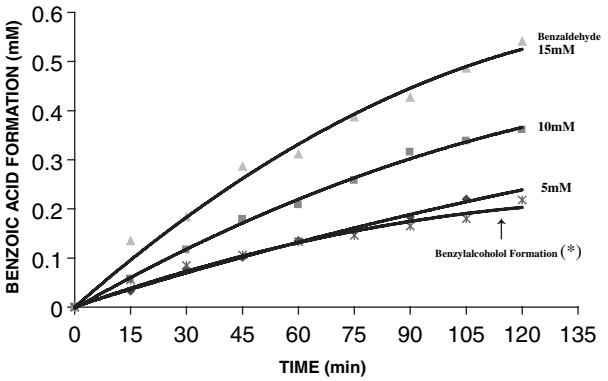


Fig. 5. Dismutation of benzaldehyde by HpCAD. The data shows the formation of benzoic acid as a function of time, at different starting concentrations of benzaldehyde (15, 10 and 5 mM). After addition of the enzyme, aliquots were removed at 15, 30, 45, 60, 75, 90, 105 and 120 min, and the amount of benzoic acid formed was estimated. The formation of benzyl alcohol is also shown at 5 mM benzaldehyde (*). Results are expressed as the means of duplicate measurements.

approximating to equilibrium. The complexity of this behaviour precludes the determination of a meaningful value for the inhibitor constant (K_i).

Dismutation

A number of alcohol dehydrogenases have been reported to catalyse the dismutation of an aldehyde to equimolar concentrations of the corresponding alcohol and carboxylic acid [30–34]. The HpCAD was found to oxidize benzaldehyde to benzoic acid utilizing $NADP^+$ as a coenzyme (Fig. 5). Through dismutation, the benzyl alcohol and benzoic acid products were produced in equimolar concentrations. The K_m for the dismutation of benzaldehyde was approximately 31 mM and the k_{cat} was approximately 2.5 s^{-1}.

Discussion

Alcohol metabolism by the gastric pathogen *H. pylori* has received little attention with the exception of a few reports that hypothesize that aldehyde production may have a role in pathogenesis [19–27]. Therefore, the aim of this study was to investigate a putative CAD from *H. pylori* and to characterize the enzyme in terms of its substrate specificity, its ability to dismutate aldehydes and to determine its subcellular localization to gain a better understanding of its role in the metabolism of alcohols and aldehydes.

The HpCAD gene product was overproduced in *E. coli*, transformed with the pET-HP1104 construct. The enzyme was purified to homogeneity using metal chelate chromatography and had a specific activity of 24 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ towards ethanol. SDS/PAGE analysis of the purified HpCAD showed a single band of 42.5 kDa and the molecular mass from size exclusion chromatography was estimated to be 50 kDa. From these data, we conclude that the enzyme is a monomer. Most previously characterized CADs were found to be dimeric, although monomeric forms have been isolated from *Eucalyptus gunnii* and *Phaseolus vulgaris* [2,35]. In the absence of dithiothreitol the enzyme had a tendency to form higher molecular mass aggregates as determined by gel filtration chromatography.

Subcellular localization studies demonstrated that the CAD was present in the cytosolic fraction of all *H. pylori* strains tested. A small amount of immunoreactivity was detectable in the total envelope fraction. This latter observation must be interpreted with caution, as it is possible that the total envelope fraction contains a small amount of cytosolic material. In contrast, a significant proportion of the CAD expressed by *M. bovis* BCG is found in the cell envelope (10–20%) [8].

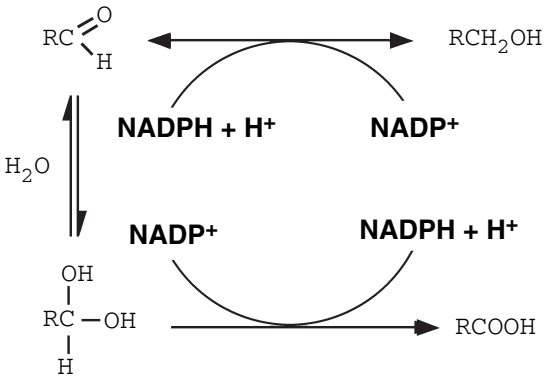
Substrate specificity analysis demonstrated that the HpCAD had a preference for aromatic aldehydes and alcohols. Furthermore, HpCAD was found to reduce aldehyde substrates that are used by plant CADs for the biosynthesis of lignin (e.g. cinnamaldehyde and coniferylaldehyde). Aliphatic and aromatic aldehydes were also reduced by the enzyme and cinnamaldehyde had the highest k_{cat}/K_m value. Having confirmed the functional activity of HP1104 as a CAD enzyme we propose that the gene encoding HP1104 be designated *cad*. This designation is further supported by the presence of several sequence motifs present in the HpCAD sequence which are common in zinc-binding medium chain dehydrogenases [9]: the putative 'catalytic zinc' ligands present at Cys42, His64 and Cys160; the pattern '$GX_{1-3}GX_{1-3}G$' which appears in the nucleotide binding region as Gly184, Gly186 and Gly189; and the four 'structural zinc' ligands at Cys95, Cys98, Cys101 and Cys109. Finally, a Ser48 is present which may play a role in the removal of the proton from alcohol molecules during the catalytic process is also present [9].

Comparison of the substrate specificity between CADs from different organisms is difficult due to variations in the alcohol and aldehyde substrates employed by different research groups. Furthermore, high substrate inhibition, where it occurs, can make specificity studies complicated, as use of a single substrate concentration may not accurately reflect relative activities if that concentration were at an inhibitory level. However, a comparison of k_{cat}/K_m values recorded for *M. bovis* BCG, *S. cerevisiae* and *Arabidopsis thaliana*

CADs with cinnamyl alcohol and aldehyde as substrates show that HpCAD is more efficient at utilizing these substrates [8–10,36]. The HpCAD enzyme also had a marked preference for the coenzyme NADP(H), with little or no activity towards NAD(H). Similar coenzyme preference was attributed to the Ser212 residue that Lauvergeat *et al.* found was responsible for determining the coenzyme specificity of *E. gunnii* CAD [37]. Larroy *et al.* also reported a Ser residue at position 211 in *S. cerevisiae*, as opposed to an Asp residue commonly found in ADH enzymes with a preference for NAD(H) as a coenzyme [9]. Sequence analysis showed there was a conserved Ser218 residue in *H. pylori* (strains 26695 and J99) which is also present in *C. jejuni*. In general, sequence alignments showed the strongest identity between CADs from *H. pylori* 26695 and *H. pylori* J99 (96%), *C. jejuni* (63%) and *M. bovis* BCG (42%).

Marked high substrate inhibition was observed in both the oxidative and reductive directions for HpCAD. The enzyme was more sensitive to high substrate inhibition when the reaction was assayed in the direction of aldehyde reduction, with such inhibition becoming apparent at 250 μM cinnam aldehyde. Similar high substrate inhibition was previously identified for a CAD from *Eucalyptus* by Lauvergeat *et al.* [37].

Significantly, HpCAD is capable of dismutating benzaldehyde to form benzyl alcohol and benzoic acid. The oxidation of benzaldehyde produces NADPH, which subsequently reduces another molecule of benzaldehyde leading to dismutation (Scheme 1). Thus the formation of both alcohol and carboxylic acid products is achieved with no net change in coenzyme



Overall reaction **2** Aldehyde + H_2O = acid + alcohol

Scheme 1. Dismutation schematic. In the dismutation reaction of HpCAD, RCHO is an aldehyde, $RCH(OH)_2$ is a hydrated aldehyde, RCOOH is the corresponding carboxylic acid and RCH_2OH is the corresponding alcohol.

oxidation and so the redox potential of the environment within the cell remains unaltered. Thus, dismutation provides an important means of reducing the concentration of potentially reactive aldehydes within the bacterium. The k_{cat}/K_m for benzaldehyde dismutation is $0.08\ s^{-1}\cdot mM^{-1}$. This is comparable to the k_{cat}/K_m for the formation of acetaldehyde from the oxidation of ethanol which is $0.15\ s^{-1}\cdot mM^{-1}$. However, the k_{cat}/K_m values for dismutation cannot be directly compared with k_{cat}/K_m values for the oxidation of alcohols or reduction of aldehydes. These latter reactions involve the binding of a substrate at a single active site and display simple saturation kinetics. The dismutation reaction, by contrast, involves substrate binding twice during the catalytic cycle [29–33]. Thus V_{max} does not represent saturation of a single substrate-binding site.

In conclusion, this work confirms the assignation of HP1104 as a CAD based on our kinetic characterization of its substrate specificity and the presence of several motifs specific to this class of enzymes. Additionally, the presence of dismutase activity is significant as, to the best of our knowledge, this is the first report of such an activity for this class of enzyme. This activity may provide the pathogen with a potential means of reducing the amount of aldehydes within the bacterium. Consequently, the hypothesis implicating *H. pylori* derived aldehydes in pathogenesis [e.g. 26]. needs to be reassessed in view of these findings.

Experimental procedures

Materials

Restriction enzymes were from New England Biolabs (Herts, England). *Taq*-High Fidelity was from Roche (Basel, Switzerland). T4 DNA Ligase was from Invitrogen (Breda, the Netherlands). Bacterial media, iminodiacetic acid-Sepharose 6B fast flow, NADP, NADPH, alcohol and aldehyde substrates and IPTG were obtained from Sigma Aldrich (Sigma, Poole, Dorset, UK).

Bacterial strains and plasmids

H. pylori strains 26695 (ATCC 700392) [38], 1061 [39], N6 (clinical isolate) and G27 were a kind gift from A. Van Vliet and J. Kusters (G27 was originally from A. Covacci – all Erasmus MC, University Medical Centre, the Netherlands). *Escherichia coli* DH5α was used for cloning procedures. Genomic DNA from *H. pylori* (strains 1061, 26695 and G27) was used to amplify the *HP1104* gene by PCR. The pET 16b vector (Novagen, Darmstadt, Germany) was used to clone and overexpress the *HP1104* gene in *E. coli* BL21(DE3)plysS with a His tag on the N terminus. *E. coli* was grown at 37 °C

in LB medium supplemented with ampicillin (100 $\mu g \cdot mL^{-1}$) and chloramphenicol (34 $\mu g \cdot mL^{-1}$) to select for the desired constructs.

Cloning methods

All DNA manipulations were performed under standard conditions as described by Sambrook *et al.* [40]. The *cad* gene was amplified by PCR using genomic DNA from *H. pylori* 26695 as the template and the oligonucleotides 5′-CGCCATATGAGACAATCTAAA-3′ and 5′-CGCGGATCCATCAAACGATTTTTTCATA-3′, as the forward and reverse primers, respectively. These primers were designed to introduce an *Nde*1 site at the 5′-end and a *Bam*H1 site at the 3′-end (underlined). The PCR conditions used were those recommended by the manufacturer (Roche, Basel, Switzerland) for *Taq* High Fidelity polymerase.

The amplified PCR product containing the *HP1104* gene was cloned into the pET 16b vector (Novagen; all pET vectors are derived from the plasmid pBR322). The resulting construct was named pET-HP1104. The construct was sequenced in both directions (DNA sequencing facility, University of Cambridge, UK) to verify that no mutations were introduced by the PCR reaction.

Purification of the *HP1104* gene product

Over production of the recombinant HpCAD was achieved in *E. coli* BL21(DE3)plysS. Cells harbouring pET-HP1104 were grown to $D_{600} = 0.6$, in LB media containing ampicillin (100 $\mu g \cdot mL^{-1}$) and chloramphenicol (34 $\mu g \cdot mL^{-1}$). Production of HpCAD was induced by addition of 1 mM isopropyl thio-β-D-galactoside, followed by incubation at room temperature, to minimize inclusion body formation. After 14 h, the cells were harvested by centrifugation at 5000 *g*, for 30 min at 4 °C. For protein purification, the cells from a 600 mL culture were resuspended in 30 mL of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris/HCl, pH 7.9) and sonicated on ice for 3× 5 min (Soniprep 150, Sanyo). The resulting cell lysate was centrifuged at 5000 ***g*** for 1 h at 4 °C, and the supernatant filtered (0.45 μm) prior to loading onto a nickel-charged iminodiacetic acid column. The unbound material was eluted using 10 column volumes of binding buffer and six column volumes of wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris/HCl, pH 7.9). The recombinant CAD protein was then eluted over seven column volumes with elution buffer (500 mM imidazole, 0.5 M NaCl, 20 mM Tris/HCl, pH 7.9).

SDS/PAGE was performed essentially as described by Laemmli [41] to monitor the purity of each fraction. Proteins were visualized by Coomassie blue staining. The purified protein was dialysed against 75 mM sodium phosphate buffer (pH 7.5) containing 5 mM dithiothreitol (dithiothreitol). Protein concentrations were determined by the Bradford method [42]. A polyclonal antibody was produced in a New Zealand White rabbit with an emulsion of purified recombinant HpCAD in Freund's complete adjuvant, using subcutaneous immunization and following standard procedures at the Bio Resource Unit, Trinity College. The polyclonal anti-HpCAD Igs were affinity purified as required from preparative Western blots of the purified recombinant protein as described by Harlow and Lane [43].

Native molecular mass determination

The relative molecular mass of the purified enzyme was determined using a Superdex 75-HR gel filtration column equilibrated with 75 mM sodium phosphate buffer (pH 7.5) containing 5 mM dithiothreitol, using an AKTA FPLC system (Amersham Pharmacia, Uppsala, Sweden). A standard curve was constructed using albumin, ovalbumin, chymotrypsinogen A and ribonuclease A (Amersham Pharmacia). CAD samples (0.2 mg) were applied at a flow rate of 1 $mL \cdot min^{-1}$.

Enzyme assays

The kinetic parameters were determined spectrophotometrically at 37 °C using an Agilent 8453 diode array spectrophotometer (Agilent Technologies, Palo Alto, CA, USA). The purified enzyme was assayed both for the reduction of aldehydes (forward reaction) and the oxidation of alcohols (reverse reaction). The activities towards different aldehydes were assayed in reaction mixtures (2 mL) containing 75 mM sodium phosphate buffer (pH 7.5) with 0.5 mM NADPH. The decrease in NADPH absorbance at 340 nm was followed to assess the enzymatic activity towards the aldehydes. The reduction of cinnamaldehyde and coniferylaldehyde was followed at 366 and 400 nm, respectively. The molar extinction coefficients (ε) used (pH 7.5) were: $\varepsilon_{340} = 6.22\ mM^{-1} \cdot cm^{-1}$ and $\varepsilon_{366} = 3.3\ mM^{-1} \cdot cm^{-1}$ for NADPH [44], although more accurate extinction coefficients have been determined under defined conditions [45]. The extinction coefficient used for coniferylaldehyde was $\varepsilon_{400} = 4.7\ mM^{-1} \cdot cm^{-1}$ [8–10]. The activities with alcohols were measured in a final volume of 2 mL in 75 mM sodium phosphate buffer (pH 7.5) containing 2 mM $NADP^+$. The formation of NADPH at 340 nm was followed for most alcohol substrates. The oxidation of cinnamyl alcohol was determined at 366 nm and coniferyl alcohol at 400 nm [8–10]. The steady-state parameters were determined by fitting the initial rates to the Michaelis–Menten equation using the ENZFITTER program.

High substrate inhibition studies

The initial rate of $NADP^+$ reduction was determined at 37 °C in 75 mM sodium phosphate buffer (pH 7.5) with

varying concentrations of $NADP^+$ and fixed concentrations of propanol at which high substrate inhibition occurred. The initial rates of $NADP^+$ reduction were determined at 100, 125, 150, 175 and 200 mM propanol.

Dismutation–benzaldehyde oxidation

Assays for aldehyde dismutation were carried out using aliquots of the reaction mixture solution removed and analysed on a Nova-Pak C18 (3.9 × 150 mm) HPLC column using the method described by Shearer *et al.* [46]. The assays (1 mL) were carried out in 75 mM sodium phosphate buffer (pH 7.5) containing 2 mM $NADP^+$ at 37 °C in the presence of various amounts of benzaldehyde. The reaction was quenched by addition of the mixture to the mobile phase (acetonitrile/acetic acid/water, 30 : 1 : 69, v/v/v) of the HPLC system. The composition of the reaction mixtures was determined using a Millipore Waters (Mississauga, Canada) liquid chromatography UV detector set at 254 nm [46]. Assays were performed in duplicate.

Subcellular localization

The subcellular fractions of *H. pylori* were obtained as described previously [47]. Briefly, *H. pylori* was grown for 48 h on Columbia agar plates containing 7% (v/v) horse blood. The bacteria were harvested and resuspended in 20 mM Tris (pH 7.5). The cells were lysed by sonication and the total membrane fraction collected by centrifugation (40 000 ***g***, 30 min, 4 °C). Membranes were resuspended in 20 mM Tris (pH 7.5) containing 2% (w/v) sodium lauryl sarcosine and incubated at room temperature for 30 min. Outer membranes were collected by centrifugation (40 000 ***g***, 30 min, 4 °C) and washed three times with Milli Q water (Millipore, Mississauga, Canada). The remaining supernatant was used as the inner membrane enriched fraction. SDS/PAGE and Western blotting were used to identify the subcellular localization of HpCAD. Blots were probed with affinity-purified rabbit anti-(HpCAD polyclonal IgG) Ig.

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