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Assessment of a putative proton relay in *Arabidopsis* cinnamyl alcohol dehydrogenase catalysis

Choonseok Lee, Diana L. Bedgar, Laurence B. Davin and Norman G. Lewis*

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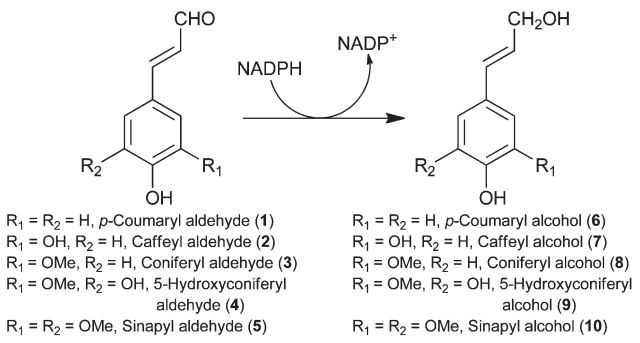
Extended proton relay systems have been proposed for various alcohol dehydrogenases, including the *Arabidopsis thaliana* cinnamyl alcohol dehydrogenases (AtCADs). Following a previous structural biology investigation of AtCAD5, the potential roles of three amino acid residues in a putative proton relay system, namely Thr49, His52 and Asp57, in AtCAD5, were investigated herein. Using site-directed mutagenesis, kinetic and isothermal titration calorimetry (ITC) analyses, it was established that the Thr49 residue was essential for overall catalytic conversion, whereas His52 and Asp57 residues were not. Mutation of the Thr49 residue to Ala resulted in near abolition of catalysis, with thermodynamic data indicating a negative enthalpic change (ΔH), as well as a significant decrease in binding affinity with NADPH, in contrast to wild type AtCAD5. Mutation of His52 and Asp57 residues by Ala did not significantly change either catalytic efficiency or thermodynamic parameters. Therefore, only the Thr49 residue is demonstrably essential for catalytic function. ITC analyses also suggested that for AtCAD5 catalysis, NADPH was bound first followed by *p*-coumaryl aldehyde.

Introduction

Cinnamyl alcohol dehydrogenases (*CAD*, EC 1.1.1.195) from vascular plants can catalyze *in vitro* the reversible conversion of *p*-coumaryl (**1**), caffeyl (**2**), coniferyl (**3**), 5-hydroxyconiferyl (**4**) and sinapyl (**5**) aldehydes into the corresponding alcohols (**6–10**) (Fig. 1), with the forward reaction being favoured.[1] In *Arabidopsis thaliana*, the first plant species whose genome was sequenced,[2] 17 genes were provisionally annotated as *CAD*s by The Arabidopsis Information Resource (TAIR).[3] Of the 9 putative *Arabidopsis thaliana CAD* genes with highest homology to *bona fide Nicotiana tabacum* and *Pinus taeda* CADs,[1,3] only AtCAD4 and AtCAD5 proteins were CADs proper based on both *in vitro* highest catalytic activity[1] and *in vivo* studies using the *A. thaliana* double mutant *cad4*/*cad5*.[4,5] The remaining 8 putative CADs had very low homology (0.9–1.6% similarity) to the *N. tabacum* and *P. taeda* CADs, and these proteins also lacked catalytic zinc.[1]



$R_1 = R_2 = H$, *p*-Coumaryl aldehyde (**1**)
$R_1 = OH$, $R_2 = H$, Caffeyl aldehyde (**2**)
$R_1 = OMe$, $R_2 = H$, Coniferyl aldehyde (**3**)
$R_1 = OMe$, $R_2 = OH$, 5-Hydroxyconiferyl aldehyde (**4**)
$R_1 = R_2 = OMe$, Sinapyl aldehyde (**5**)

$R_1 = R_2 = H$, *p*-Coumaryl alcohol (**6**)
$R_1 = OH$, $R_2 = H$, Caffeyl alcohol (**7**)
$R_1 = OMe$, $R_2 = H$, Coniferyl alcohol (**8**)
$R_1 = OMe$, $R_2 = OH$, 5-Hydroxyconiferyl alcohol (**9**)
$R_1 = R_2 = OMe$, Sinapyl alcohol (**10**)

Fig. 1 Enzymatic reactions catalysed by AtCAD5 in presence of NADPH.

Cinnamyl alcohol dehydrogenases belong to the medium-chain dehydrogenase/reductase family,[6] and a structural biology study of the AtCAD5 apo-enzyme and its binary complex with $NADP^+$ was reported by Youn *et al.*[7] In terms of the putative CAD mechanism, there are currently two possible mechanisms for related alcohol dehydrogenases, using either an extended proton relay (transfer)[8] or a penta-coordinated zinc system.[9] The extended proton relay mechanism assumes that a water molecule bound to tetrahedrally coordinated catalytic zinc is displaced by an incoming alcohol substrate and that this species is maintained during catalysis.[8]

That proposed mechanism was based on the study of the ternary complex of horse liver alcohol dehydrogenase (HLADH), crystallized in the presence of NAD^+ and *p*-bromobenzyl alcohol, where it was reported that the catalytic zinc ion was ligated to the alcohol oxygen *via* a tetrahedrally coordinated complex (Fig. 2a).[8,10–13] In addition, an extended hydrogen bonding system was reported between the hydroxyl group of the substrate alcohol coordinated to zinc ion and Ser48, between Ser48 and nicotinamide ribose (O2′), and between nicotinamide ribose (O2′) and His51 in the ternary complex of HLADH, whereas in the apo-enzyme the hydrogen bond

Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340, USA. E-mail: lewisn@wsu.edu; Fax: +1-509-335-8206; Tel: +1-509-335-2682

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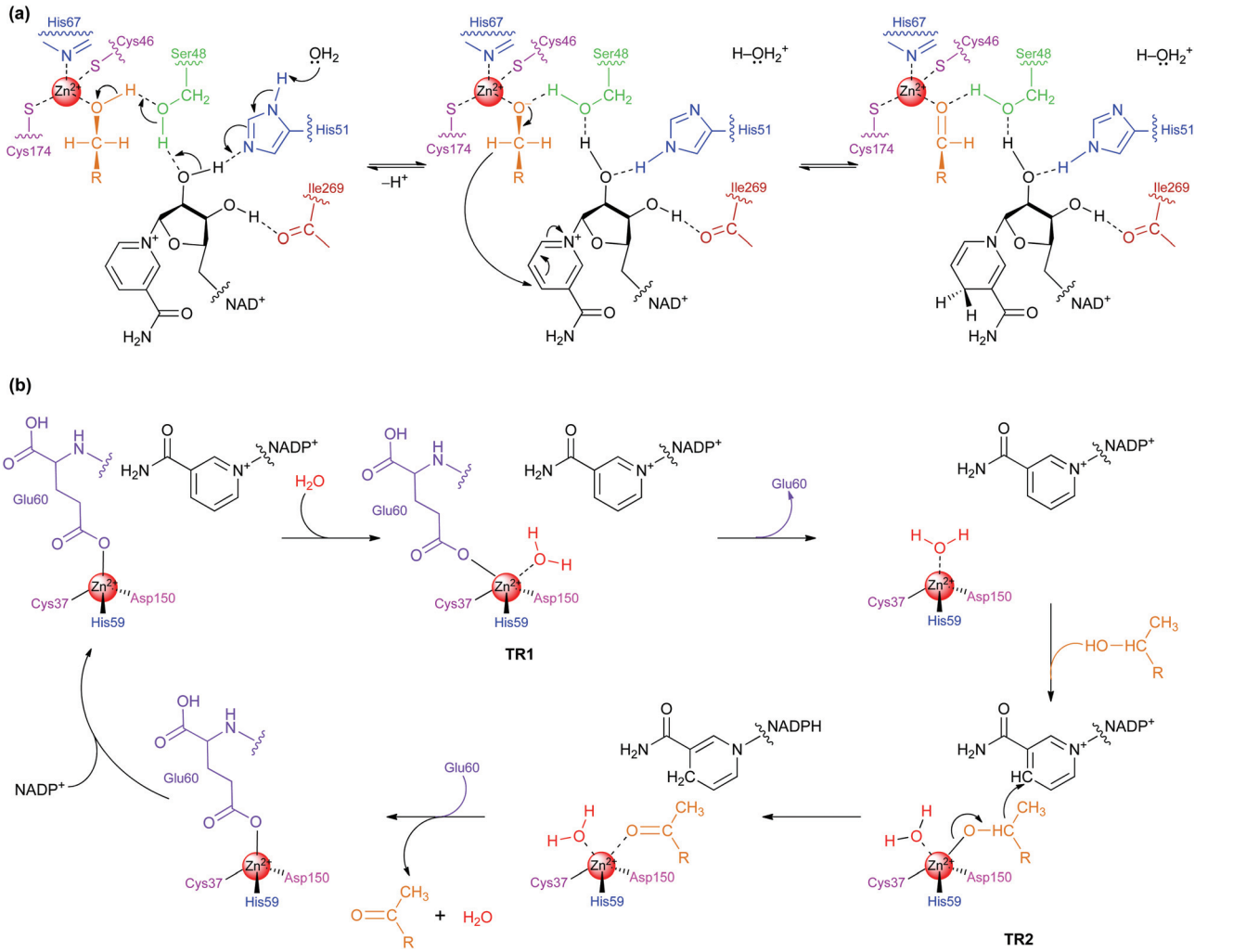

Fig. 2 Two proposed catalytic mechanisms for alcohol dehydrogenases: (a) Proton relay system in horse liver alcohol dehydrogenase (HLADH); adapted and redrawn from Eklund *et al.*[8] (b) Penta-coordinated zinc system in *Thermoanaerobacter brockii* alcohol dehydrogenase (TbADH); adapted and redrawn from Kleifeld *et al.*[9] **TR1**: the first transient complex, **TR2**: the second transient complex.

system was not observed.[8] In contrast to HLADH, however, a ternary complex with substrate and cofactor is not available for AtCAD5 thus far.[7]

In the alternative model (Fig. 2b), binding of the alcohol substrate putatively results in formation of a penta-coordinated zinc complex,[9] as deduced from analysis of results obtained with a *Thermoanaerobacter brockii* alcohol dehydrogenase (TbADH) using extended X-ray absorption fine structure, pre-steady state kinetics, and density functional theory calculation approaches. In that study, two distinct penta-coordinate intermediates were postulated as being formed during the catalytic cycle. Specifically, the following was envisaged: when $NAPD^+$ is bound to TbADH, the tetra-coordinated zinc ion species is maintained, and the first penta-coordinated zinc ion transient complex is formed by addition of a water molecule. The penta-coordinated zinc ion species is then converted back to a tetra-coordinated zinc ion complex by dissociation of the ligated Glu60 residue. Binding of the incoming alcohol substrate regenerates a penta-coordinated zinc ion complex, the second transient species. The catalytic cycle is envisaged to be completed through dissociation of the water molecule and product, and re-ligation of Glu60 to regenerate the original tetra-coordinated zinc ion species, *i.e.* returning the enzyme to its resting state.[9] Site-directed mutagenesis however, gave a Glu60Ala mutant whose catalytic efficiency was only reduced *circa* four fold relative to the wild type enzyme.[9] Overall, this mechanism (Fig. 2b) does not require an extended proton relay transfer as for the one described for HLADH (Fig. 2a).

In terms of developing an understanding of overall CAD catalysis, the first report was that describing molecular modelling of *Eucalyptus gunnii* CAD2 against HLADH.[14] This study suggested that its putative substrate binding region in the active site included residues Ile95, Tyr113, Trp119 and Phe298 substituted from the corresponding residues Phe93, Phe110, Leu116 and Ile318 of HLADH as reviewed in Lewis *et al.*[15] Both Trp119 and Phe298 residues[14] were proposed as providing a "molecular sandwich", whereby the phenolic ring of the

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substrate is stabilized, and in an orientation enabling abstraction of the pro-*R* hydride from NADPH during the reductive step.[16] In the reverse reaction, the 9-pro-*R* hydrogen of coniferyl alcohol (**8**) is removed.[17] In addition, Ser212/Arg217 residues in *E. gunnii* CAD2, and Asp223/Lys228 in HLADH, were predicted to be involved in cofactor binding in this model,[14] with the Ser212 residue further confirmed to be closely related to NADPH binding through its site-directed mutant, Ser212Asp.[18]

The putative three-dimensional molecular structure of *Eucalyptus gunnii* CAD2 also suggested that both residues, Ser49 and His52, served in a proton relay system in a comparable way as for Ser48 and His51 residues in HLADH.[14] In a somewhat analogous manner, following analysis of the binary complex of AtCAD5, the corresponding residues, Thr49 and His52, were also putatively involved in a proton relay, together with Asp57 (Fig. 3), with the latter being postulated *via* substrate modelling of the binary complex of AtCAD5.[7]

Isothermal titration calorimetry (ITC) is now being widely used to investigate protein–protein, protein–nucleic acid and protein–lipid interactions, drug design and enzyme kinetics.[19] In particular, this approach is very effective for the study of how proteins interact with either substrates, cofactors or ligands through analysis of binding stoichiometry (N), binding enthalpy (ΔH), dissociation constant K_d, binding entropy (ΔS) and Gibbs free energy of binding (ΔG).[19–28] Furthermore, for site-directed mutated proteins, ITC can effectively be utilized to compare the behaviour of native (recombinant) proteins and their mutated forms.

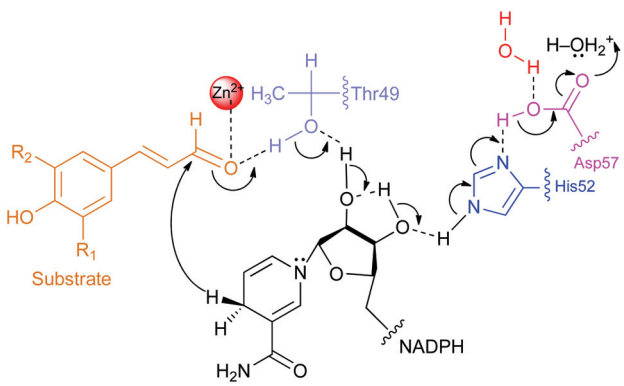

Fig. 3 Putative proton shuttle mechanism for AtCAD5. The dotted lines indicate the putative hydrogen bonds/proton relay. [Adapted and redrawn from Youn *et al.*[7]] For R_1, R_2 explanation, see Fig. 1.

In the investigation herein, site-directed mutagenesis and ITC were used to further evaluate and assess the validity of a putative extended proton relay mechanism in AtCAD5,[7] including study of the effects of mutating putative key amino acid residues on substrate and cofactor binding, as well as catalytic turnover. Additionally, this study examined the order of substrate/co-factor binding during AtCAD5 catalysis as an ordered bi–bi mechanism had been suggested by Wyrambik and Grisebach.[29]

Results and discussion

Residues Thr49, His52 and Asp57 of AtCAD5 were individually mutated into Ala through PCR (see Table 1 for each primer set used). It was envisioned that these mutations would establish which of the three amino acid residues were potentially involved in a proton relay (Fig. 3). After removal of parental methylated and hemi-methylated DNA with *Dpn* I restriction enzyme, each mutated DNA was individually transformed into One shot® Top 10 *Escherichia coli* cells.[30] After selecting each mutated clone, mutations at each position were confirmed by sequencing, with each construct next transformed into *E. coli* cells for protein expression. Except for use of the Fast Protein Liquid Chromatography system, the purification method employed was as described in Kim *et al.*[1] Each recombinant protein was obtained from an imidazole-containing eluent (from ~120 to 180 mM in 20 mM Tris-HCl, 500 mM NaCl, pH 7.9) followed by concentration/buffer exchange in 20 mM Tris buffer (pH 7.5). Each enzyme was purified to apparent homogeneity (data not shown) and used to obtain kinetic and thermodynamic parameters.

Kinetic analyses of AtCAD5 and its site-directed mutants

Kinetic parameters for AtCAD5 and its site-directed mutants were initially obtained using the Michaelis–Menten equation[31] with *p*-coumaryl aldehyde (**1**) employed as a substrate (Table 2).

In our hands, the native (recombinant) AtCAD5 gave a K_m 13 μM, V_{max} 88.1 *p*kat μg^{-1}, and catalytic turnover of 6.8 s^{-1}. That is, the substrate, *p*-coumaryl aldehyde (**1**), was bound very tightly and was efficiently converted into the product, *p*-coumaryl alcohol (**6**). These data were thus in good agreement with our previous study of AtCADs.[1]

As indicated earlier, in our previous study of the binary complex with AtCAD5, Thr49 was proposed to be hydrogen-

Table 1 Sequences of forward (F) and reverse (R) primers used for site-directed mutagenesis

Primers	Sequence (5′ → 3′)
AtCAD5 T49A F	GCTGTGGAATCTGCCACGCGGATCTTCATCAAACTA
AtCAD5 T49A R	TAGTTTGATGAAGATCCGCGTGGCAGATTCCACAGC
AtCAD5 H52A F	GAATCTGCCACACCGATCTTGCGCAAACTAAAAATGATCTTG
AtCAD5 H52A R	CAAGATCATTTTTAGTTTGCGCAAGATCGGTGTGGCAGATTC
AtCAD5 D57A F	CAAACTAAAAATGCGCTTGGCATGTCT
AtCAD5 D57A R	AGACATGCCAAGCGCATTTTTAGTTTG

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Table 2 Kinetic parameters for AtCAD5 and its site-directed mutants with *p*-coumaryl aldehyde (**1**) as substrate in presence of NADPH

Enzyme	K_m (μM)	V_{max} (*p*kat μg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
AtCAD5	13 ± 1.9	88.1 ± 3.5	6.8 ± 0.3	523 000 (±46 000)
T49A	114 ± 11.3	0.33 ± 0.01	0.026 ± 0.001	230 (±18)
H52A	117 ± 6.9	518.9 ± 16.5	40.2 ± 1.3	345 000 (±8700)
D57A	36 ± 3.1	957.7 ± 31.0	74.2 ± 2.4	2 061 000 (±102 000)

bonded to the O2′ of the nicotinamide ribose moiety and thus was considered a potential residue in the putative proton transfer mechanism.[7] Participation of this residue in the overall enzymatic reaction was established, *via* analysis of the Thr49Ala mutant which was found to be essentially catalytically inactive. The affinity of the substrate, *p*-coumaryl aldehyde (**1**), was though still quite high (K_m 114 μM), whereas the V_{max} was 0.33 *p*kat μg^{-1} and the catalytic turnover number was 0.026 s^{-1}. Thus, the Thr49Ala mutation specifically caused a near complete loss in catalytic efficiency.

On the other hand, kinetic analysis of the AtCAD5 H52A recombinant protein gave very different results. The data obtained established that this residue was not essential for catalysis overall, even though it was provisionally envisioned earlier as being involved in the proton relay *via* hydrogen bonding to O3′ of the nicotinamide ribose. In our hands, substrate affinity was still relatively high (K_m 117 μM) for *p*-coumaryl aldehyde (**1**), the V_{max} increased ~6 fold (518.9 *p*kat μg^{-1}) with a catalytic turnover of 40.2 s^{-1}. In an analogous manner, the D57A mutant gave a catalytically active enzyme with K_m 36 μM for *p*-coumaryl aldehyde (**1**). However, the V_{max} also apparently increased ~11 fold with the overall catalytic turnover larger by ~4 fold over the native (recombinant) protein. Therefore, the kinetic analysis of AtCAD5 D57A demonstrated that the Asp57 residue was also not essential for overall catalysis in AtCAD5. Taken together, only the Thr49 residue can be considered essential for the overall catalytic mechanism of AtCAD5.

There is an earlier precedent for this observation with the $\beta_1\beta_1$ isoenzyme of human liver alcohol dehydrogenase (HuLADH).[32] Its site-directed mutant His51Gln (corresponding to His52 in AtCAD5) also had 2 (at pH 7, 8 and 9) or 3 (at pH 10) times higher V_{max} in the mutant, relative to wild type enzyme, this being rationalized as due to less tight binding of NADH thereby enabling it to be more readily dissociated.[32–34] Accordingly, the mutation of His52 or Asp57 may facilitate release of $NADP^+$ during catalysis.

Isothermal titration calorimetry analyses of AtCAD5 and its site-directed mutants

ITC measurements at constant temperature and pressure were carried out to further investigate the binding properties of AtCAD5 and its site-directed mutants using either *p*-coumaryl aldehyde (**1**) or NADPH. Thermodynamic parameters which included dissociation constant K_d ($K_d = 1/K_a$; K_a, binding association constant) and enthalpy of binding (ΔH) were thus obtained. The Gibbs free energy of binding was calculated from $\Delta G = -RT \ln K_a$, where R is the gas constant and T the temperature. The binding entropy was obtained from $\Delta S = (\Delta H - \Delta G)/T$.[19]

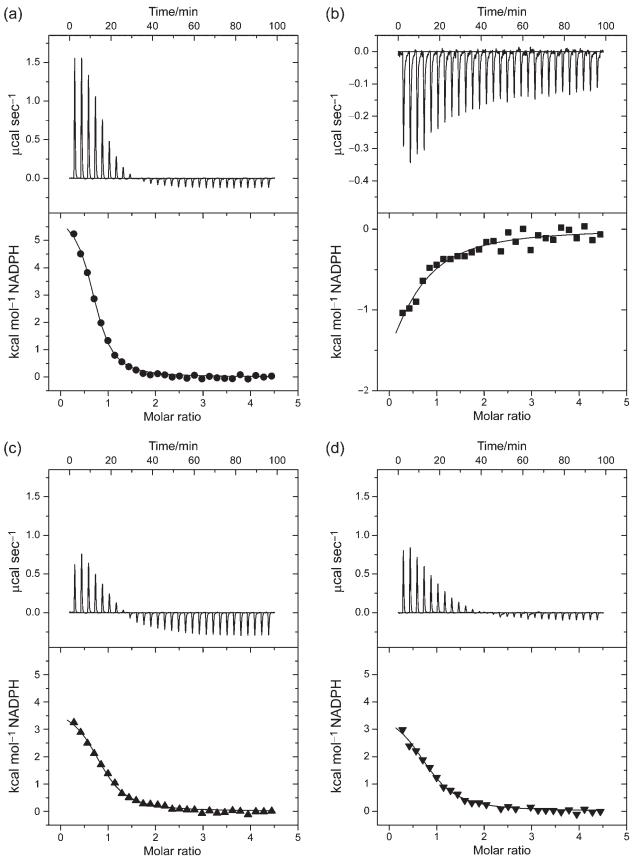

Fig. 4 Isothermal titration calorimetry analyses of AtCAD5 (a), AtCAD5 T49A (b), AtCAD5 H52A (c) and AtCAD5 D57A (d) with NADPH. The top panels show raw data of the heat pulses resulting from each titration, whereas the bottom panels show the integrated heat normalized per mole of NADPH as a function of the molar ratio (NADPH concentration/enzyme homodimer concentration).

Binding of *p*-coumaryl aldehyde (**1**) to AtCAD5 and its mutants, however, yielded inconclusive results due to very low heat generation for all enzymes tested, suggesting that *p*-coumaryl aldehyde (**1**) either binds very weakly or cannot bind to the free enzyme (data not shown).

In contrast, significant heat changes were observed upon binding of NADPH to AtCAD5 or its mutants (Fig. 4). Specifically, the NADPH binding data obtained were fitted best to a model that specifies one set of sites, with N values from 0.7 to 0.8 obtained (Table 3), these being indicative of ~1 : 1 stoichiometry per enzyme dimer. These data thus indicate that one

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Table 3 Thermodynamic parameters for NADPH binding to AtCAD5 and its mutants

Protein	N^a	K_d (μM)	ΔG (kJ mol^{-1})	ΔH (kJ mol^{-1})	$T\Delta S$ (kJ mol^{-1})
AtCAD5	0.7 (±0.02)	3.5 (±0.1)	−31.7 (±0.1)	26.0 (±0.3)	57.7 (±0.3)
AtCAD5 T49A	0.8 (±0.002)	37.2 (±11.6)	−25.8 (±0.9)	−12.0 (±5.7)	13.8 (±6.3)
AtCAD5 H52A	0.8 (±0.02)	7.1 (±1.1)	−29.9 (±0.4)	17.1 (±0.7)	47.0 (±0.3)
AtCAD5 D57A	0.8 (±0.02)	8.2 (±0.5)	−29.5 (±0.1)	15.6 (±0.2)	45.1 (±0.1)

[a] *N* indicates binding stoichiometry of NADPH binding per enzyme homodimer.

molecule of NADPH is bound to AtCAD5, a homodimer,[7] or its site-directed mutants. Valencia *et al.* also reported that a putative CAD-like homolog from *Saccharomyces cerevisiae* binds one NADPH molecule per homodimer, this being rationalized to only one functional subunit probably being available for catalysis.[35] Similarly, *Pseudomonas putida* tartrate dehydrogenase (TDH), a homodimer, also reportedly showed a 1 : 1 stoichiometry of NADH binding per enzyme dimer.[36]

The ITC data for binding of NADPH to AtCAD5 is summarized in Table 3, with a binding isotherm and the fitted data shown in Fig. 4A. NADPH was found to bind to AtCAD5 with a dissociation constant of ~3.5 μM. Formation of the binary complex AtCAD5·NADPH is endothermic with a ΔH of 26.0 kJ mol^{-1} (Table 3, Fig. 4a).

The essentially catalytically inactive T49A mutant was next examined (Table 3 and Fig. 4b). The thermodynamics of NADPH binding were affected by the T49A mutation: the K_d of NADPH increased by >10 fold, indicating a significant decrease in binding affinity, with formation of the binary complex now being exothermic (ΔH of −12.0 kJ mol^{-1}) and driven by a less favourable $-T\Delta S$. As shown in the putative modelled ternary complex of AtCAD5 (Fig. 3),[7] the Thr49 is proposed to interact with the substrate and NAD(P)H and is also located near to the catalytic Zn^{2+}. Replacement of Thr49 with Ala thus disrupts the interaction between this amino acid residue, catalytic Zn^{2+}, and putatively NADPH and the substrate, the net effect being decreased NADPH binding affinity (Table 3) and a near complete abolition of catalytic activity (Table 2).

In agreement with the kinetic data, the thermodynamic properties of NADPH binding for H52A and D57A mutant proteins were very similar to that of WT (Table 3 and Fig. 4c,d). Specifically, NADPH binding yielded a K_d of 7.1 μM, ΔH of 17.1 kJ mol^{-1} and $T\Delta S$ of 47.0 kJ mol^{-1} for the H52A mutant, and K_d of 8.2 μM, ΔH of 15.6 kJ mol^{-1} and $T\Delta S$ of 45.1 kJ mol^{-1} for the D57A mutant. Thus, this implies that mutation of His52 or Asp57 to alanine did not very significantly affect binding of NADPH to the corresponding protein in contrast to mutation of Thr49 to Ala.

In sum, NADPH binding to AtCAD5 and its mutants is a thermodynamically favourable processes as indicated by the negative ΔG values (between −25.8 and −31.7 kJ mol^{-1}).

Titration of the binary complex AtCAD5·NADPH with *p*-coumaryl aldehyde (**1**), followed by the enzymatic conversion into the corresponding alcohol (**6**), was next investigated at 25 °C. The overall process was found to be exothermic (Fig. 5a), in contrast to formation of the binary complex AtCAD5·NADPH which was endothermic (Fig. 4a). However, when NADPH was injected to the cell containing both AtCAD5 and *p*-coumaryl aldehyde (**1**), a very different profile was observed (Fig. 5b). First, an endothermic reaction occurred corresponding to binding of NADPH to AtCAD followed by an exothermic response due to the catalytic conversion of **1** into **6**. Together, these results further confirm that the cofactor binds first to the enzyme followed by the aldehyde substrate. Similar results were observed with the mutant enzymes (data not shown).

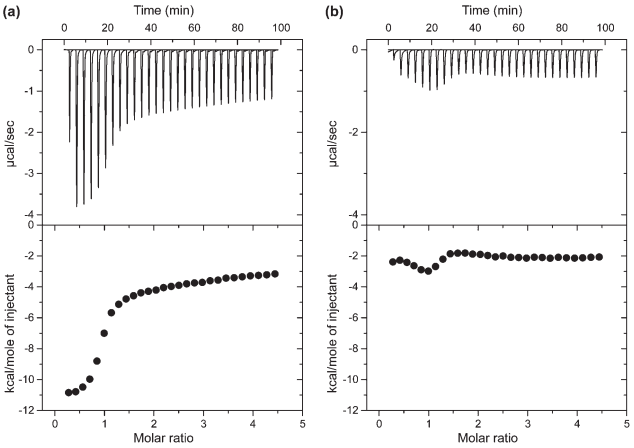

Fig. 5 Isothermal titration calorimetry (ITC) analysis of AtCAD5. (a) Titration at 25 °C of AtCAD5 and NADPH with *p*-coumaryl aldehyde (**1**). (b) Titration at 25 °C of AtCAD5 and *p*-coumaryl aldehyde (**1**) with NADPH. The top panel show raw data of the heat pulses resulting for each titration, whereas the bottom panels show the integrated heat normalized per mole of injectant as a function of the molar ratio (injectant concentration/enzyme homodimer concentration).

Conclusions

In the putative proton relay system for AtCAD5,[7] three residues, Thr49, His52 and Asp57, were provisionally implicated in its catalysis as well as for other dehydrogenases.[37,38] However, from the kinetic and ITC data of these site-directed mutations in AtCAD5, all three residues were apparently not essential for catalysis. Notably, only mutation of Thr49 residue caused a loss in catalytic activity for AtCAD5, this being further verified by a significant decrease in binding affinity, a negative ΔH from a positive ΔH in AtCAD5 and a decrease of $T\Delta S$ in NADPH binding.

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However, mutation of His52 and Asp57 residues did not affect catalytic efficiency, but, in contrast, the turnover numbers and maximum velocities of the His52Ala and Asp57Ala mutants increased. Moreover, binding properties of AtCAD5 H52A and D57A remained largely unchanged.

From the ITC analyses, it was also concluded that NADPH binds first to AtCAD5 followed by the substrate.

Taken together, the data provide no evidence for an extended proton relay system.

Experimental

Materials

p-Coumaryl (**1**) aldehyde and *p*-coumaryl alcohol (**6**) were synthesized as in Kim *et al.*[1] NADPH (tetrasodium salt) was obtained from Sigma. Water (Optima® LC/MS) and acetonitrile (Optima® LC/MS) were obtained from Fisher Scientific, and glacial AcOH (HPLC grade) was procured from Mallinckrodt Baker.

The QuikChange® XL Site-Directed Mutagenesis Kit was purchased from Stratagene, whereas pTrcHis2-TOPO vector and One Shot TOP 10 competent *E. coli* cells were from Invitrogen. BugBuster® protein extraction reagent, Benzonase® nuclease and rLysozyme™ solution, were obtained from Novagen. The POROS 20 metal chelate resin was from Applied BioSystems.

Instrumentation

Polymerase chain reactions (PCR) were carried out using a PTC-0220 DNA engine dyad Peltier thermal cycler (MJ Research). Recombinant protein purification was carried out on a Fast Protein Liquid Chromatography (FPLC, Amersham Pharmacia Biotech) system using a column packed with POROS 20 metal chelate resin.

Reversed-phase chromatography employed an Ultra Performance Liquid Chromatography® system (UPLC, Waters) equipped with a BEH shield RP 18 column (Waters); flow rate of 0.3 ml min^{-1}; detection at 280 nm. The solvent system consisted of a concave gradient (Waters curve #8) of CH_3CN : 3% AcOH (v/v) in H_2O from 5 : 95 to 40 : 60 between 0 and 8.30 min.

Isothermal titration calorimetry measurements used a VP-ITC microcalorimeter (MicroCal Inc.).

Site-directed mutagenesis

AtCAD5, cloned into the pTrcHis2-TOPO vector, was used as previously described.[1] Primers were designed and synthesized (Invitrogen) to individually change Thr49, His52 and Asp57 into Ala (Table 1). Site-directed mutagenesis was carried out using a QuikChange® XL Site-Directed Mutagenesis Kit following the manufacturer's instructions with PCR performed as follows: 95 °C for 1 min, 18 cycles at 95 °C for 50 s, 60 °C for 50 s, 68 °C for 4 min, and 68 °C for 7 min. Parental plasmid DNA was next digested with *Dpn* I restriction enzyme (10 units), at 37 °C for 1 h, with the resulting mutated plasmid DNA individually transformed into One Shot TOP 10 competent *E. coli* cells. Transformants were selected on LB medium containing carbenicillin (100 μg ml^{-1}). Positive clones were next subjected to sequencing to confirm single mutations.

Expression and purification of AtCAD5 and its mutants

Clones of AtCAD5 and its mutants were individually inoculated in 5 ml of LB medium containing carbenicillin (100 μg ml^{-1}) and incubated at 37 °C overnight. Next, 250 ml of LB medium containing carbenicillin (100 μg ml^{-1}) was inoculated with 2.5 ml of the corresponding 5 ml culture. The *E. coli* cells were incubated at 37 °C at 230 rpm until an OD_{600} ~ 0.8 was reached, with induction then initiated by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.2 mM final concentration). After incubation at 20 °C for 24 h, the cells were harvested by centrifugation, and stored at −20 °C until needed.

Frozen pellets were individually thawed, resuspended in 10 ml of BugBuster protein extraction reagent containing Benzonase® nuclease (250 units) and rLysozyme™ solution (10 000 units) at room temperature for 20 min. Each lysed solution was centrifuged (4300 × *g*, 25 min), with the supernatant filtered through a 0.2 μm syringe filter (Pall).

Recombinant protein purification was carried out at 4 °C on a POROS 20 metal chelate column (60 × 10 mm) pre-equilibrated in binding buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 5 mM imidazole) at a flow rate of 4 ml min^{-1}. Aliquots (~10 ml) of each recombinant protein preparations were next loaded onto the affinity column, with the latter first washed with 10% elution buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 500 mM imidazole) in binding buffer, after which a gradient from 10 to 100% was applied in 25 min. Fractions eluting between ~120 and ~180 mM imidazole were combined and concentrated with Centricon Plus-70 (Amicon), diluted with 20 mM Tris-HCl buffer (pH 7.5), and concentrated to 10–20 mg ml^{-1} (Centricon Plus-70, Amicon). Final purity was confirmed by SDS-PAGE (4–15% acrylamide, BioRad) with silver staining (Amersham Bioscience Application Note). Protein concentrations were determined by the Bradford method[39] using γ-globulin as standard.

Assay of AtCAD5 and its mutants

Standard assays were as described in Kim *et al.*[1] with initial velocity kinetics data carried out in presence of 50 ng ml^{-1} AtCAD5, 105 ng ml^{-1} AtCAD5 H52A, 100 ng ml^{-1} AtCAD5 D57A, and 14.3 μg ml^{-1} AtCAD5 T49A. Final *p*-coumaryl aldehyde (**1**) concentrations were: 0.76–80 μM for AtCAD5, 0.76–200 μM for AtCAD5 H52A and D57A, and finally, 7.6–480 μM for AtCAD5 T49A, respectively. Assays, carried out in quadruplicate, were initiated by enzyme addition, incubated at 30 °C for 1 min (2 min for AtCAD5 T49A) and stopped by addition of glacial AcOH (10 μl). Aliquots (10 μl) from each assay mixture were subjected to reversed phase UPLC as described in the Instrumentation section. For each enzyme preparation, kinetic data was calculated using Origin 7.5 (OriginLab).

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Isothermal titration calorimetry (ITC)

Titrations were carried at constant pressure and at 30 °C (for cofactor/substrate binding) or at 25 °C (for order of binding study). Twenty nine injections (10 μl each) at 200 s intervals were performed, with the first injection not used for data analysis. The stirring speed was 300 rpm.

For cofactor/substrate binding, the sample cell (1.4 ml) was filled with either AtCAD5 or its mutants at a final concentration of 50 μM (of homodimer) in HEPES buffer (20 mM, pH 6.8) containing 100 mM NaCl (Buffer A). The injection syringe (250 μl) contained NADPH or *p*-coumaryl aldehyde (**1**) (1 mM) in Buffer A. Titrations were carried out in triplicate. A control titration was carried out by injecting ligand in Buffer A in order to determine the dilution/mixing heat which was then subtracted for each experiment prior to data analysis.

To study the order of binding, *p*-coumaryl aldehyde (**1**, 1 mM) was titrated into a cell containing AtCAD5 (50 μM, homodimer) and NADPH (75 μM) in Buffer A. In another experiment, NADPH (1 mM) was titrated into a cell containing AtCAD5 (50 μM, homodimer) and *p*-coumaryl aldehyde (**1**, 75 μM). ITC analyses were then carried out as above.

The Origin 5.0 software (MicroCal) was utilized to obtain dissociation constant K_d, and binding enthalpy (ΔH) for each.

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