Engineering the nucleotide coenzyme specificity and sulphydryl redox sensitivity of two stress-responsive aldehyde dehydrogenase isoenzymes of Arabidopsis thaliana

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INTRODUCTION

Plants are subject to diverse environmental constraints, which cause an accumulation of ROS (reactive oxygen species) to reach excessive and imbalanced levels. ROS cause disruption of the cellular machinery and homeostasis [1]. The irreversible damage of ROS can be ascribed to two aspects. First, ROS directly interact with a variety of molecules including amino acids, proteins, nucleic acids and membrane lipids, disrupting cell metabolism and integrity [2]. The second component of ROS-mediated injury is related to lipid peroxidation of polyunsaturated fatty acids, which leads to chain breakage. Thus reactive breakdown products are generated including saturated and unsaturated hydrocarbons, hydroxyl acids and aldehydes, which in turn propagate ROS-mediated oxidation thereby exacerbating cellular damage. Given the high reactivity of ROS and the toxicity of the products generated, plants have evolved a variety of defence strategies. The first line of defence is the regulation of the steady-state level of ROS, which involves the avoidance of ROS formation and the detoxification of reactive products. It includes several enzymatic scavengers such as superoxide dismutase, ascorbate peroxidase, glutathione peroxidase, glutathione transferase, superoxide reductase and hydrogen peroxide oxidoreductase, and a number of molecules like ascorbate, tocopherols, carotenoids and glutathione, that function as anti-oxidants contributing to protection [1]. The second line of defence is the repair of ROS-mediated damage using molecules like thioredoxins and glutaredoxins.

The harmful effect of surplus amounts of aldehydes is well established [3]. A recent study shows that lipid peroxide-derived aldehydes, especially highly electrophilic \( \alpha,\beta \)-unsaturated aldehydes, are involved in aluminium toxicity in plants and suppression by 2-alkenal reductase provides an efficient defence mechanism [4]. Despite their toxicity when accumulated in excess, aldehydes are ubiquitous molecules that take part in different physiological processes. For example, induction of hydroperoxide lyase expression leads to enhancement of \( \alpha \)-olefin formation after pathogen infection and increases resistance [5]. \( \alpha \)-aldehydes, such as (Z)-3-hexenal, (E)-2-hexenal or \( n \)-hexanal, act directly as fungicidal and bactericidal compounds [6,7] that induce the synthesis of the phytoalexin camalexin and subsequent defence responses in Arabidopsis thaliana [8]. Green leafy volatiles, including (Z)-3-hexenal, may play a key role in plant–plant signalling and plant–insect interactions [9]. (Z)-3-hexenal activates defence responses during herbivore attack, such as transient jasmonic acid biosynthesis and the release of other volatiles, thereby priming plants to respond towards subsequent herbivore attack and simultaneously triggering defence reactions in neighbouring plants. Therefore it is crucial to maintain the balance between physiologically essential and deleterious levels. One of the major detoxification processes of excessive aldehydes is their oxidation into the corresponding carboxylic acid, which

Lipid peroxidation is one of the consequences of environmental stress in plants and leads to the accumulation of highly toxic, reactive aldehydes. One of the processes to detoxify these aldehydes is their oxidation into carboxylic acids catalyzed by NAD(P)\(^+\)-dependent ALDHs (aldehyde dehydrogenases). We investigated kinetic parameters of two Arabidopsis thaliana family 3 ALDHs, the cytosolic ALDH3H1 and the chloroplastic isoform ALDH3I1. Both enzymes had similar substrate specificity and oxidized saturated aliphatic aldehydes. Catalytic efficiencies improved with the increase of carbon chain length. Both enzymes were also able to oxidize \( \alpha,\beta \)-unsaturated aldehydes, but not aromatic aldehydes. Activity of ALDH3H1 was NAD\(^+\)-dependent, whereas ALDH3I1 was able to use NAD\(^+\) and NADP\(^+\). An unusual isoleucine residue within the coenzyme-binding cleft was responsible for the NAD\(^+\)-dependence of ALDH3H1. Engineering the coenzyme-binding environment of ALDH3I1 elucidated the influence of the surrounding amino acids. Enzyme activities of both ALDHs were redox-sensitive. Inhibition was correlated with oxidation of both catalytic and non-catalytic cysteine residues in addition to homodimer formation. Dimerization and inactivation could be reversed by reducing agents. Mutant analysis showed that cysteine residues mediating homodimerization are located in the N-terminal region. Modelling of the protein structures revealed that the redox-sensitive cysteine residues are located at the surfaces of the subunits.

Key words: aldehyde dehydrogenase (ALDH), coenzyme specificity, enzymatic activity, oxidative stress, thiol regulation, site-directed mutagenesis.
is catalysed by ALDHs (aldehyde dehydrogenases; EC 1.2.1.3) using NAD⁺ and NADP⁺ as coenzymes [10].

ALDHs are very diverse: some are substrate specific, whereas others react with a broad array of substrates. In addition, some use either NAD⁺ or NADP⁺ as a coenzyme whereas others can use both. By 2002 over 550 distinct genes encoding ALDHs had been characterized, evolving from a few type 1 enzymes through green algae and mosses to diverse classes in eukaryotes. Eukaryotic ALDHs are classified into more than 20 families [11]. ALDHs are localized in various subcellular compartments. Crystal structures of ALDHs from different families have been resolved. All have a common architecture and are stabilized by intra-molecular hydrogen bonds; some such as family 1 and 2 enzymes are enzymatically active as homotetramers, whereas others like family 3 ALDHs function as homodimers [12–14]. The A. thaliana genome contains 14 genes encoding ALDHs localized in different subcellular compartments [15]. They belong to nine protein families ranging from substrate-specific to variable-substrate enzymes. Members of ALDH gene families 3, 5 and 7 have been reported to respond to environmental stress conditions [15–17].

ALDHs participate in different pathways in plants, but their precise physiological role is often still unclear. Plant ALDHs have gained increased attention since the maize mitochondrial ALDH2B2 was identified as the rfa2 gene, which is a nuclear restorer of cytoplasmic male sterility [18,19]. A mitochondrial family 2 ALDH in rice may be responsible for acetaldehyde detoxification during re-aeration after submergence [20]. Nair et al. [21] showed that the ref1 (reduced epidermal fluorescence 1) mutant of Arabidopsis is caused by a mutation in the ALDH2C4 gene (The Arabidopsis Information Resource accession number AT3g24503), which is involved in the biosynthesis of ferulic and sinapic acid. A recent study suggests that ALDH2B4 is involved in the pyruvate dehydrogenase bypass pathway in Arabidopsis [22]. Overexpression of ALDH3H1 in A. thaliana improved stress tolerance when plants were exposed to osmotic, oxidative or heavy metal stress [23]. Similarly, ectopic expression of an ALDH7 gene (The Arabidopsis Information Resource accession number AT3g09100) in rice alleviates ROS accumulation [25], which could indicate that the two additional cysteine residues affect the analyses, a stop codon was inserted downstream of the 3' EcoRI site by mutagenesis PCR.

For ALDH3I1 expression, an EcoRI DNA fragment (1470 bp, 470 amino acids, nucleotides 195–1665) was subcloned into the pET28a expression vector (Novagen) yielding a fusion protein of 528 amino acids with an N-terminal His-tag. The ALDH3H1 fragment was amplified by PCR from a cDNA clone (GenBank® accession number AJY72122) with the following primers (restriction sites are shown in bold): sense primer, position 183–204, 5'-CTGTGAAGAAGGAAATCTCGAT-3'; antisense primer, position 1590–1596, 5'-AGAGCATTTGAATTCTAGAAAT-3'. As a result of this cloning strategy, the C-terminal part of the ALDH3H1 amino acid sequence is 24 amino acids longer and contains two additional cysteine residues. To avoid the possibility that the two additional cysteine residues affect the analyses, a stop codon was inserted downstream of the 3' EcoRI site by mutagenesis PCR. For ALDH3I1 expression, an EcoRI/XhoI DNA fragment (1470 bp, 490 amino acids, nucleotides 195–1665) was subcloned into the pET28a expression vector yielding a fusion protein of 534 amino acids with both an N-terminal and a C-terminal His-tag. The ALDH3I1 fragment was amplified by PCR from a cDNA clone (GenBank® accession number AJ306961), with the following primers (restriction sites are shown in bold): sense primer, position 178–200, 5'-CTTATCGGTTG-3'; antisense primer, position 1681–1685, 5'-CTTATCGGTTG-3'. The recombinant protein thus lacks most of the chloroplastic signal peptide, except for 8 amino acids including the cysteine residue at amino acid position 55 (Supplementary Figure S2 at http://www.BiochemJ.org/bj/434/bj4340459add.htm). The ALDH3H1 and ALDH3I1 expression constructs were transformed into Escherichia coli strain BL21 (DE3).

Purification of soluble recombinant ALDH3H1 and ALDH3I1 proteins was performed by metal ion affinity chromatography on His-tag binding columns (Sigma–Aldrich) under native conditions [16] with the following changes. Bacterial cultures were pre-incubated for 30 min at 24°C and induced by adding 0.1 mM IPTG (isopropyl β-D-thiogalactopyranoside) for 3 h at 24°C. Bacterial cell pellets were resuspended in extraction buffer [50 mM Heps/NaOH (pH 7.4), 300 mM NaCl, 10% (v/v) glycerol, 0.1% Triton X-100 and 1.5 mM β-ME (2-mercaptoethanol; freshly added)], supplemented with 10 mg/ml lysozyme and 5 mM imidazole. Purified proteins were eluted in 250 µl fractions with extraction buffer containing 250 mM imidazole. Eluted peak protein fractions (e.g. fraction 4 for ALDH3H1 and fraction 5 for ALDH3I1; Supplementary Figure S1 at http://www.BiochemJ.org/bj/434/bj4340459add.htm) were
selected and adjusted to 50% (v/v) glycerol, 1 mM PMSF, 0.5 mM NAD\(^+\) and 6 mM DTT (dithiothreitol) to stabilize
the enzymes before storage at −80 °C for further use. The
peak fractions always had a comparable protein yield and activity (Supplementary Table S2 at http://www.BiochemJ.org/434/bj4340459add.htm). Protein concentrations were
determined with the Bradford protein assay (Bio-Rad) using
BSA as a standard. Purity of eluted proteins was verified by
SDS/PAGE (12% gel) analysis and immunoblotting with anti-
ALDH antisera [25].

Oxidation and reduction of recombinant ALDH3H1 and ALDH3I1
Purified proteins were oxidized by incubation with 50 μM CuCl\(_2\) for 1 h at room temperature (22 °C). Subsequently, the redox
state of the enzymes was assessed by non-reducing SDS/PAGE
(10% gel). Prior to the oxidation, protein fractions were dialysed
against 50 mM Hepes (pH 7.4) using PD-10 desalting columns
(GE Healthcare). To reduce oxidized ALDH3H1 and ALDH3I1,
fractions were incubated for 1 h at room temperature with
various concentrations of DTT or GSH (Roth). Following the
re-reduction, the redox state of the proteins was confirmed by
non-reducing SDS/PAGE (10% gel).

Quantification of free sulphydryl groups
Purified ALDH3H1 and ALDH3I1 proteins were oxidized by
incubation with 50 μM CuCl\(_2\) for 2 h at room temperature
and then samples were collected at different time points.
The ALDH activities of the collected samples were measured
immediately and free thiol groups were simultaneously
determined spectrophotometrically based on Ellman’s test
[36] using DTNB [5,5′-dithiobis(2-nitrobenzoic acid); Sigma–
Aldrich]. 2 μl of 20 mM DTNB was added to 18 μl of each ALDH
sample, mixed with 780 μl of 0.1 M potassium phosphate buffer
(pH 7.4) and then incubated for 20 min at room temperature
to allow colour development. The absorbance of released thiophenol
anions (TNB\(^−\)) was determined at 412 nm. Data were plotted as
the percentage of the remaining free sulphydryl groups in the
oxidized fractions compared with those initially present in the
reduced form.

Construction of the ALDH mutants
All mutants were prepared according to the QuikChange\textsuperscript{®}
site-directed mutagenesis protocol (Stratagene) with mega-
primers carrying the desired mutations. Protein expression
and purification of the mutated enzymes were performed as
described above. All mutants, the corresponding primers and
the amino acid positions in the recombinant enzymes are
listed in Supplementary Table S1 (http://www.BiochemJ.org/
bj/434/bj4340459add.htm). Mutants are numbered according to
the amino acid position in the native proteins. Cysteine mutants
Cys45Ser, Cys247Ser and Cys253Ser were produced for the
enzyme ALDH3H1 and cysteine mutants Cys114Ser, Cys142Ser,
Cys286Ser, Cys310Ser and Cys316Ser were produced for the
enzyme ALDH3I1. Coenzyme affinity studies were performed
using Ile200Val and Ile200Gly mutants for ALDH3H1 and a
Val263Ile mutant for ALDH3I1.

ALDH3H1 and ALDH3I1 enzyme activity and determination of
kinetic constants
Enzymatic activity assays and determination of apparent \(K_m\)
and \(V_{max}\) values were performed as described [16]. The assay
buffer contained 100 mM sodium pyrophosphate at the respective
pH-optima for ALDH3H1 and ALDH3I1, 1.5 mM NAD(P)\(^+\)
(Roche) and various concentrations of propionaldehyde (Merck),
hexanal, octanal, nonanal, dodecanal, \(trans-2\)-hexenal, \(trans-2\)-
oneanal (Sigma–Aldrich) or 4-hydroxynonenal (Calbiochem).
Coenzyme specificity was determined using hexanal and \(trans-2\)-
oneanal, as aldehyde substrates at saturating concentrations,
and various concentrations of NAD(P)\(^+\). Hexanal was used in
these experiments because of its higher solubility in aqueous
solution. All kinetic parameters are reported as means ± S.E.M. of
at least three independent experiments. Enzyme specific activities
are expressed as either μmol of NADH/min per mg of protein
or μmol of NADPH/min per mg of protein. Catalytic efficiency
is expressed as \(V_{max}/K_m\) (app) (μmol NAD(P)H·min\(^{−1}\)·mg\(^{−1}\)
per μM aldehyde) \times 10\(^5\).

RESULTS
Expression and purification of recombinant Arabidopsis ALDH3H1
and ALDH3I1 proteins
To examine the biochemical features of ALDH enzymes,
ALDH3I1 and ALDH3H1 recombinant proteins were purified.
Generally, approx. three times more soluble ALDH3H1 protein
was obtained than ALDH3I1 protein from a comparable amount
of bacterial culture. Supplementary Table S2 summarizes the
overall yield of both recombinant enzymes in typical purification
experiments. Supplementary Figures S1(A) and S1(C) show
typical purification profiles for both proteins. The identity of
the eluted proteins was confirmed by immunoblot analysis
(Supplementary Figures S1B and S1D). The SDS/PAGE gel
shows the purity of the protein fractions, resulting in major
protein bands for ALDH3H1 and ALDH3I1 of approx. 56 and
58 kDa respectively, corresponding to the monomeric subunits.
The purified ALDH3I1 migrated during SDS/PAGE as a tight
doublet, which may be ascribed to the presence of intramolecular
disulfide bonds generated by oxidation of the subunit molecules.

Kinetic properties of Arabidopsis ALDH3H1 and ALDH3I1
Activities of purified recombinant ALDH proteins were measured
across a broad pH range using 1 mM hexanal and 1.5 mM NAD\(^+\)
as substrates. The pH-optima for ALDH3H1 and ALDH3I1 were
8.0 and 9.0 respectively (Supplementary Figure S1), which were
used in all further enzyme assays.

The preferred substrates were determined using saturated
aliphatic aldehydes, unsaturated aliphatic aldehydes and the
hydroxylated aldehyde 4-hydroxynonenal with NAD\(^+\) as
coenzyme. Medium- to long-chain saturated aldehydes (C\(_6\)
to C\(_{12}\)) were preferred as substrates, whereas the short-chain
aldehyde propanal was a weak substrate, as determined by the
catalytic efficiency \(V_{max}/K_m\). Dodecanal was the best substrate
with catalytic efficiency values of 4831 for ALDH3H1 and
15028 for ALDH3I1 and 15028 for ALDH3H1 and a
K\(_m\) of 5 μM and 1.3 μM respectively
(Table 1). Although the α,β-unsaturated aldehydes \(trans-2\)-
hexenal, \(trans-2\)-nonenal and 4-hydroxynonenal were substrates
for both enzymes with regard to K\(_m\) values, catalytic efficiencies
for the unsaturated aldehydes were lower than for the saturated
aldehydes of the same carbon chain length. The results suggest
that saturated aldehydes are preferred over unsaturated aldehydes,
irrespective of chain length.

Coenzyme preference was analysed in the presence of easily
soluble substrate, like the saturated hexanal and the
unsaturated aldehyde \(trans-2\)-nonenal (Table 2). A comparison of the
kinetic constants for NAD\(^+\) and NADP\(^+\) shows that NAD\(^+\)
was the preferred coenzyme for both enzymes. ALDH3I1 was also able to use NADP+ to oxidize both substrates, whereas ALDH3H1 was strictly NAD+ specific. This was confirmed for other aldehydes as substrates (results not shown). The coenzyme preference of ALDH3I1 was substrate dependent. Whereas the oxidation of trans-2-nonenal in the presence of NADP+ was characterized by $K_v$ values comparable with those found for NAD+, the $K_v$ value for hexanal was approx. 26-fold higher with NADP+ than the corresponding value obtained with NAD+ as the nucleotide coenzyme (Table 2A).

**Coenzyme specificity is modified by single amino acid substitutions**

Alignment of amino acid sequences revealed that ALDH3H1 and ALDH3F1 are exceptions within the family 3 ALDH (Figure 1A). ALDH3H1 and ALDH3F1 contain an isoleucine residue instead of a valine at a central position in the coenzyme-binding site (Figure 1B). This amino acid position is opposite the $\beta$-hydroxyl of the adenine ribose of NAD+ [37] (Figures 1A and 1B). The isoleucine residue in this location could explain why ALDH3H1 is only able to use NAD+ as coenzyme, whereas other family 3 ALDHs function with NADP+ or NAD+. To examine this hypothesis, the isoleucine residue at position 200 was replaced by valine or by glycine to yield ALDH3H1<sub>Val200Val</sub> and ALDH3H1<sub>Glu200Glu</sub>. The mutated ALDH3H1 proteins were expressed in E. coli and purified to homogeneity. Purification profiles were similar to the wild-type ALDH3H1.

The kinetic properties and coenzyme specificities of the ALDH3H1 mutants were determined using hexanal as a substrate at saturating concentrations. Mutating the isoleucine residue to the smaller residues of valine or glycine changed the distance between the amino acid at position 200 and Glu<sup>149</sup>. This single amino acid exchange altered coenzyme specificity (Table 2). For ALDH3H1<sub>Glu200Val</sub> the distance as determined by structure modelling from Val<sup>200</sup> across the cleft to Glu<sup>149</sup> is approx. 9.23 Å (1 Å = 0.1 nm), which is nearly 1.4 Å longer than the distance in the wild-type ALDH3H1 (7.85 Å) (Figure 1D), but similar to ALDH3I1 (9.22 Å) or the rat ALDH3A1 (9.19 Å). The latter two enzymes are exceptions within the family 3 ALDH and ALDH3I1 (9.15 Å) (Figures 1C and 1E–G). Similarly, the ALDH3H1<sub>Glu200Val</sub> mutant acquired the ability to use NADP+ as coenzyme with a $K_{vNADP^+}$ in the same range as the wild-type ALDH3H1. The relative catalytic efficiency of ALDH3H1<sub>Glu200Val</sub> for NADP+ was ~1% of that for NAD+ as deduced from the ratio ($V_{\text{max NADP+}} / K_{vNADP^+}$)($V_{\text{max NADP+}} / K_{vNADP^+}$). A slight increase was observed in $K_{vNADP^+}$, namely 496 μM for the mutated enzyme compared with 421 μM for the non-mutated ALDH3H1, but there was no significant negative effect on $V_{\text{max}}$.

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**Table 1** Kinetic parameters of recombinant *Arabidopsis* ALDH3H1 and ALDH3I1 proteins

<table>
<thead>
<tr>
<th>Substrate</th>
<th>ALDH3H1 $K_v$ (μM)</th>
<th>$V_{\text{max}}$ (μmol NAD - min⁻¹ · mg⁻¹)</th>
<th>$V_{\text{max}}$/K_v</th>
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<tbody>
<tr>
<td>Propionaldehyde</td>
<td>510 ± 59</td>
<td>7.3 ± 2.7</td>
<td>16</td>
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<tr>
<td>Hexanal</td>
<td>71 ± 12</td>
<td>12 ± 0.6</td>
<td>165</td>
</tr>
<tr>
<td>Octanal</td>
<td>29 ± 4</td>
<td>18 ± 5.5</td>
<td>617</td>
</tr>
<tr>
<td>Nonanal</td>
<td>8 ± 2</td>
<td>19.2 ± 4.1</td>
<td>2318</td>
</tr>
<tr>
<td>Dodecanal</td>
<td>5 ± 1</td>
<td>23.9 ± 1.4</td>
<td>4831</td>
</tr>
<tr>
<td>trans-2-Hexenal</td>
<td>180 ± 24</td>
<td>2.4 ± 0.16</td>
<td>13.3</td>
</tr>
<tr>
<td>trans-2-Nonenal</td>
<td>3 ± 0.7</td>
<td>2.9 ± 0.18</td>
<td>938</td>
</tr>
<tr>
<td>4-Hydroxynonenal</td>
<td>40.3 ± 8</td>
<td>1.4 ± 0.02</td>
<td>34</td>
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<table>
<thead>
<tr>
<th>Substrate</th>
<th>ALDH3I1 $K_v$ (μM)</th>
<th>$V_{\text{max}}$ (μmol NAD - min⁻¹ · mg⁻¹)</th>
<th>$V_{\text{max}}$/K_v</th>
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<tr>
<td>Propionaldehyde</td>
<td>8053 ± 1331</td>
<td>10.1 ± 1.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Hexanal</td>
<td>111 ± 29</td>
<td>17.3 ± 2.8</td>
<td>156</td>
</tr>
<tr>
<td>Octanal</td>
<td>24 ± 10</td>
<td>16.6 ± 3.9</td>
<td>701</td>
</tr>
<tr>
<td>Nonanal</td>
<td>7 ± 1</td>
<td>20 ± 4.4</td>
<td>3028</td>
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<tr>
<td>Dodecanal</td>
<td>1.3 ± 0.2</td>
<td>188 ± 1.9</td>
<td>15028</td>
</tr>
<tr>
<td>trans-2-Hexenal</td>
<td>151 ± 18</td>
<td>1.5 ± 0.1</td>
<td>9.7</td>
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<tr>
<td>trans-2-Nonenal</td>
<td>5.5 ± 1.9</td>
<td>1.6 ± 0.5</td>
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<tr>
<td>4-Hydroxynonenal</td>
<td>21 ± 1.3</td>
<td>0.6 ± 0.04</td>
<td>28</td>
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</table>

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**Table 2** Kinetic parameters of recombinant wild-type (A) and mutated (B) *Arabidopsis* ALDH3H1 and ALDH3I1 comparing NAD+ and NADP+ as coenzymes

<table>
<thead>
<tr>
<th>Coenzyme</th>
<th>ALDH3H1 $K_v$ (μM)</th>
<th>$V_{\text{max}}$ (μmol NAD - min⁻¹ · mg⁻¹)</th>
<th>$V_{\text{max}}$/K_v</th>
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<tr>
<td>NAD&lt;sup&gt;+&lt;/sup&gt;</td>
<td>421 ± 23.5</td>
<td>18.4 ± 1.7</td>
<td>43.8</td>
</tr>
<tr>
<td>NADP&lt;sup&gt;+&lt;/sup&gt;</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>NAD&lt;sup&gt;+&lt;/sup&gt;</td>
<td>119 ± 25</td>
<td>2.8 ± 0.8</td>
<td>23</td>
</tr>
<tr>
<td>NADP&lt;sup&gt;+&lt;/sup&gt;</td>
<td>na</td>
<td>na</td>
<td>na</td>
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</table>

<table>
<thead>
<tr>
<th>Mutant</th>
<th>ALDH3H1&lt;sub&gt;Val200Val&lt;/sub&gt; $K_v$ (μM)</th>
<th>$V_{\text{max}}$ (μmol NAD - min⁻¹ · mg⁻¹)</th>
<th>$V_{\text{max}}$/K_v</th>
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<tbody>
<tr>
<td>ALDH3H1&lt;sub&gt;Val200Val&lt;/sub&gt;</td>
<td>496 ± 4</td>
<td>19.8 ± 1.8</td>
<td>40</td>
</tr>
<tr>
<td>ALDH3H1&lt;sub&gt;Glu200Glu&lt;/sub&gt;</td>
<td>3218 ± 54</td>
<td>17.4 ± 1.8</td>
<td>5.4</td>
</tr>
<tr>
<td>ALDH3H1&lt;sub&gt;Glu200Val&lt;/sub&gt;</td>
<td>126 ± 2</td>
<td>12.0 ± 0.2</td>
<td>95.2</td>
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<tr>
<th>Mutant</th>
<th>ALDH3I1 $K_v$ (μM)</th>
<th>$V_{\text{max}}$ (μmol NADPH - min⁻¹ · mg⁻¹)</th>
<th>$V_{\text{max}}$/K_v</th>
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<tr>
<td>NAD&lt;sup&gt;+&lt;/sup&gt;</td>
<td>71 ± 5</td>
<td>14.1 ± 1.3</td>
<td>200</td>
</tr>
<tr>
<td>NADP&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1868 ± 101</td>
<td>3.0 ± 0.2</td>
<td>1.6</td>
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<tr>
<td>NAD&lt;sup&gt;+&lt;/sup&gt;</td>
<td>53 ± 6</td>
<td>1.3 ± 0.2</td>
<td>24</td>
</tr>
<tr>
<td>NADP&lt;sup&gt;+&lt;/sup&gt;</td>
<td>87 ± 10</td>
<td>0.5 ± 0.1</td>
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Biochemical features of Arabidopsis family 3 aldehyde dehydrogenases

Figure 1: The coenzyme-binding site among family 3 ALDHs

(A) Alignment of amino acid sequences of parts of the coenzyme-binding site among family 3 ALDHs. UniProt accession numbers are listed in brackets. Bovine: ALDH3B1 (Q1JPA0); Human: ALDH3B1 (P43353), ALDH3B2 (P48448), ALDH3A2 (P51648) and ALDH3A1 (P30838); Mouse: ALDH3B1 (Q80VQ0), ALDH3A1 (P47739) and ALDH3A2 (P47740); Rat: ALDH3B1 (Q5X42), ALDH3A2 (P30839) and ALDH3A1 (P11883); Pongo abelii (PONAB) ALDH3A2 (Q5RF60); Macaca fascicularis (MACFA) ALDH3A2 (Q5H9H); Dog (CANFA) ALDH3A1 (A3RF36); C. plantagineum (CRAPL) Cp-ALDH (Q8VXQ2); A. thaliana (ARATH) ALDH3H1 (Q70DU8), ALDH3I1 (Q8W033) and ALDH3F1 (Q70E96). The conserved glutamate and valine residues are indicated in green and the isoleucine in red. The amino acid sequences shown in blue in (A) correspond to the coenzyme binding site regions modelled in (B–G). The positions of the amino acids in the native proteins are indicated by the numbers on the left-hand and right-hand side for each sequence. (B) The positions of amino acids are shown inside the coenzyme binding cleft of ALDH3A1 and ALDH3H1. (C–G) Location of NAD⁺ and NADP⁺ adenine ribose moieties and amino acid residues in the coenzyme-binding site of the previously solved structure of ALDH3A1 from Rattus norvegicus [14] and the predicted A. thaliana ALDH3H1 and ALDH3I1. The coenzyme-binding cleft is magnified from the ribbon diagram of the crystal structure of the ALDH3A1 monomer (C and D) and the models of ALDH3H1 (E) and ALDH3I1 monomers (F and G). The binary complex ALDH3H1–NADP⁺ is not presented, because ALDH3H1 is an NAD⁺-dependent dehydrogenase. Amino acid residues discussed to be important for the coenzyme binding (see Results section) are highlighted either in green for conserved residues or in red for the unusual residue in this position, i.e. isoleucine. Coenzymes NAD⁺ (C–E) and NADP⁺ (F and G) are shown in stick representation and atoms are depicted as follows: oxygen, red; carbon, cyan; phosphorus, orange; nitrogen, blue; hydrogen atoms are hidden. Distances between residues across the coenzyme-binding cleft are indicated in blue. For ALDH3H1 and ALDH3I1, these are estimations based on the homology structure modelling. The predicted structures of ALDH3H1 and ALDH3I1 were generated by the Web-based server SWISS-MODEL [35], and rendered using PyMol.

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The ratio \( K_{\text{mNAD}}/K_{\text{mNADP}}^+ \) was 0.21 for the mutant, which reflects a higher affinity to NAD\(^+\) than to NADP\(^+\). This ratio was still 5-fold higher than it was for ALDH3I1, due to the low \( K_a \) of ALDH3I1 for NAD\(^+\), which was seven times lower than ALDH3H1.

The Ile200Gly mutation exerted the maximal effect on coenzyme binding by ALDH3H1. It caused a lower affinity for NAD\(^+\) with a \( K_{\text{mNAD}}^+ \) nearly 8-fold higher than the wild-type ALDH3H1 and approx. 6-fold higher compared with ALDH3H1\(_{\text{HeC00WV}}\), but it had little effect on \( V_{\text{max}} \). However, the relative preference for NAD\(^+\) (\( V_{\text{maxNAD}}^+/V_{\text{maxNADP}}^+ \)) decreased more than 4-fold. Simultaneously, the ability to interact with NADP\(^+\) as coenzyme increased compared with ALDH3H1\(_{\text{HeC00WV}}\). The Ile200Gly substution led to a \( K_{\text{mNADP}}^+ \) lower than the respective Michaelis constant for NAD\(^+\) and changed the coenzyme preference from NAD\(^+\) to NADP\(^+\). Whereas the wild-type enzyme was strictly dependent on NAD\(^+\), the isoüecine to valine residue mutation enabled the enzyme to use NADP\(^+\) while still preferring NAD\(^+\).

The \( K_a \) values for hexanal were similar for the mutated enzymes, i.e. ALDH3H1\(_{\text{HeC00WV}}\) and ALDH3H1\(_{\text{HeC00Gy}}\), compared with the respective wild-type enzymes (results not shown). This indicates that the interaction with the substrate was not affected by the mutation of the coenzyme-binding site. The increase in the specificity for NADP\(^+\), coupled with the decrease in the affinity for NAD\(^+\), paralells the enlargement of the coenzyme-binding-site cleft. The differential evolution of the \( K_a \) values for NAD\(^+\) and NADP\(^+\) suggests that the mutation of Ile200 to a glycine residue changed the nucleotide specificity of ALDH3H1 from NAD\(^+\)-dependence to an NADP\(^+\)-compatible ALDH, with a stronger preference for NADP\(^+\) in case of ALDH3H1\(_{\text{HeC00WV}}\).

To examine whether the presence of Ile200 was the only factor influencing the width of the coenzyme-binding cleft and consequently determining the ALDH3H1 NAD\(^+\)-dependence, the ALDH3H1 NAD(P)-binding site was engineered and an isoüecine residue was introduced to replace the orthologous Val263. This modification did not affect hexanal substrate specificity compared with the saturation kinetics of the wild-type enzyme (results not shown). To compare the coenzyme affinities of the mutated enzymes, kinetic parameters of the mutated enzyme ALDH3H1\(_{\text{Val263Ile}}\) were determined from NAD\(^+\) saturation curves after full activation with the substrate hexanal (Table 2B).

Homology structure modelling suggested that restricting the distance across the coenzyme-binding cleft in ALDH3I1 from 9.22 Å to 7.72 Å, as result of the Val263Ile substitution, should decrease the available space to accommodate the 2'-phosphate group of the ribose of NAD\(^+\). However, the ALDH3I1\(_{\text{Val263Ile}}\) variant showed a very good ability to use NADP\(^+\) as a coenzyme. The apparent \( K_{\text{mNADP}}^+ \) was reduced more than 2-fold in comparison with the wild-type enzyme, whereas the apparent \( K_{\text{mNADP}}^+ \) value for ALDH3I1\(_{\text{Val263Ile}}\) was nearly 2-fold higher than the value for the wild-type enzyme (Table 2B).

### Enzymatic activities of ALDH3H1 and ALDH3I1 are dependent on their redox states

To examine whether the enzymatic properties of ALDH3H1 and ALDH3I1 proteins are affected by their redox state, enzymatic activities were measured, with hexanal as a substrate and NAD\(^+\) as a coenzyme, under different redox conditions. Oxidation led to a decrease in enzymatic activities to less than 25–35% of the activity of the corresponding reduced forms (Figure 3). Reduction of oxidized ALDH3H1 after incubation with 10 mM DTT for 1 h resulted in a good recovery of activity to ~83% of the initial activity, but to only 44% after reduction with 10 mM GSH (Figure 3A). Oxidation of ALDH3I1 led to a loss of ~70% of the initial enzymatic activity, but could be restored to ~60% after incubation with 10 mM DTT and to 36% by treating with GSH (Figure 3B). This indicates a thiol regulation and rules out unspecific oxidation.

### ALDH inactivation is correlated with loss of sulphhydryl groups

Densiometric analysis of SDS/PAGE patterns indicated the relative amounts of ALDH monomers versus dimers in the different redox conditions. The ratio between dimers and monomers is not well correlated with the observed loss of activity (Supplementary Table S3 at http://www.BiochemJ.org/bj/434/bj4340459add.htm). Hence, dimers cannot be considered as the only inactive forms of both enzymes and inhibition of activities can also be attributed to a stepwise oxidation of other cysteine residues (catalytic and non-catalytic).

To examine whether ALDH activity during oxidation is correlated with a loss of other sulphhydryl groups, enzymatic activity was determined and free thiol groups were quantified simultaneously (see Experimental section). During oxidation with CuCl\(_2\), the release of TNB (5-thio-2-nitrobenzoic acid) revealed a negative linear correlation during the first 65 min, which reflects a constant loss of free sulphhydryl groups (Figures 4A and 4B). The fact that the ALDH activities of both enzymes were correlated with the number of sulphhydryl groups suggests a thiol-based regulation, which includes sulphhydryl-group oxidation as well as homodimerization.

### Identification of cysteine residues critical for enzymatic activities and dimer formation

To investigate which cysteine residues are involved in dimer formation or contribute to enzyme activities, cysteine residues were individually mutated to serine. The enzyme activities and dimer formation of the mutants were analysed. The native ALDH3H1 subunit contains three cysteine residues located at positions 45, 247 and 253 (Supplementary Figure S2). Three single Cys mutants were generated (see Experimental section and Supplementary Table S1). ALDH3H1\(_{\text{Cys247Ser}}\) and ALDH3H1\(_{\text{Cys247Ser}}\) mutants were as soluble as the wild-type enzyme, whereas the ALDH3H1\(_{\text{Cys245Ser}}\) mutant was less soluble. Alignment of the amino acid sequences of selected ALDH sequences shows that Cys\(^{255}\) is conserved (Supplementary Figure S2). Several studies have shown that a cysteine residue in this position is part of the active centre [38–40].

### ALDH3H1 and ALDH3I1 proteins form disulfide-linked dimers and multimers under oxidizing conditions

When the ALDH3H1 and ALDH3I1 proteins were treated with H\(_2\)O\(_2\) or CuCl\(_2\) and separated by non-reducing SDS/PAGE, proteins were generated with a molecular mass corresponding to homodimers (~112 kDa and ~116 kDa respectively) and multimeric bands of higher molecular mass (Figures 2A and 2C). This may be attributed to intermolecular disulfide bond formation. To investigate this possibility, oxidized proteins were incubated with increasing concentrations of the disulfide bond cleaving reagents DTT or GSH. Figures 2(B) and 2(D) show that dimers disappeared and, conversely, monomers were gradually recovered. The efficiency of the reduction of intermolecular disulfide bonds was higher when treated with DTT than with GSH (results not shown).
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Figure 2 Oxidation and reduction of ALDH3H1 and ALDH3I1 proteins

Recombinant ALDHs were purified as enzymatically active homodimers. Homodimers and multimers were generated by incubating the enzymes (5 μg ALDH3H1 or 10 μg ALDH3I1) for 90 min at room temperature in the presence of 50 μM CuCl2 or 1 mM H2O2. ALDH3H1 (A) and ALDH3I1 (C) proteins were separated by non-reducing SDS/PAGE (10% gel). Molecular mass is shown on the right-hand side in kDa. Oxidized ALDH3H1 (B) or ALDH3I1 (D) were incubated with increasing concentrations of DTT for 1 h at room temperature. Multimeric forms of ALDH3H1 and ALDH3I1 were separated by non-reducing SDS/PAGE (10% gel) and proteins were detected using PageBlue protein staining solution (Fermentas).

Similarly, Cys316 is part of the active centre of ALDH3I1 (Supplementary Figure S2). Mutation of Cys253 in ALDH3H1 and of Cys316 in ALDH3I1 abolished enzymatic activity and confirmed that these cysteine residues belong to the catalytic centre. Mutations of other cysteine residues led only to a partial loss of activity. In the case of ALDH3H1, the ALDH3H1Cys247Ser mutant retained approx. 90% of the wild-type catalytic activity (Figure 5A).

The cysteine residues of ALDH3I1 at positions 114, 142, 286, 310 and 316 were mutated to serine (Supplementary Table S1). All mutant enzymes were as soluble as the wild-type enzyme. Mutation of Cys114 to a serine residue led to a decrease in enzymatic activity of more than 80%, but activity did not change significantly when the cysteine residues at positions 286 or 310 were replaced by serine (Figure 5B). Although Cys55 and Cys316 in ALDH3H1 and ALDH3I1 respectively are not conserved between different ALDHs, their mutation to a serine residue caused a decrease in enzyme activity of more than 70% (Figure 5).

The cysteine residues present in ALDH3H1 allow six different possible combinations of intermolecular disulfide bonds between its subunits. However, only the Cys45Ser mutation affected the intermolecular disulfide bond formation, which suggests that Cys45 of ALDH3H1 is the redox-responsive residue required to form an intermolecular disulfide bond under oxidizing conditions (Figure 6A). Similarly, cysteines of ALDH3I1 involved in the formation of intermolecular disulfide bonds were identified. ALDH3I1 contains nine cysteine residues. Cysteine 55 and Cys114 in ALDH3I1 respectively are not present in the mature protein. Therefore the number of theoretically possible combinations for disulfide bond formation in the mature ALDH3I1 protein is 15. Only the ALDH3I1Cys114Ser mutant did not form dimers (Figure 6B), indicating that Cys114 is critical for dimerization.

Cysteine mutant analyses demonstrated that the conserved amino acids, Cys253 in ALDH3H1 and Cys316 in ALDH3I1, are critical for the catalytic activity and the N-terminal cysteine residues, Cys55 in ALDH3H1 and Cys114 in ALDH3I1, have a catalytically facilitating role, therefore their oxidation impairs the dehydrogenase activity in addition to their involvement in dimerization.

Oxidation can be reversed and enzyme activity restored

To confirm the impact of thiol-group oxidation and intermolecular disulfide bond formation on ALDH activity, the redox state...
of the different cysteine residue mutations were determined and the enzymatic activities were analysed. Oxidative treatment resulted in the partial inhibition of enzyme activity (Figure 5) and dimerization (Figures 3 and 6). The ALDH3H1Cys247Ser, ALDH3H1Cys142Ser, ALDH3H1Cys286Ser and ALDH3I1Cys310Ser dimers could be gradually reverted to monomers following incubation with sulfhydryl-reducing reagents such as GSH or DTT (Figure 7). The release of the monomers from ALDH disulfide-bond-linked homodimers was accompanied by a partial recovery in dehydrogenase activity (Figure 5). Densitometric analysis also revealed that the correlation between monomer recovery and the rate of enzyme reactivation is low (Supplementary Table S3). This is further evidence for the involvement of other oxidized thiol groups.

**DISCUSSION**

**Enzymatic properties**

The focus of the present paper was the biochemical characterization of the *Arabidopsis* family 3 ALDH enzymes ALDH3H1 and ALDH3I1, which are localized in the cytosol and chloroplasts respectively [16,25]. Both genes are highly conserved and have probably been maintained by selective pressure, indicating that the functions of the gene products are required in cytoplasm and chloroplasts [16,25]. Both genes are highly conserved and have probably been maintained by selective pressure, indicating that the functions of the gene products are required in cytoplasm and chloroplasts [16,25]. Both genes are highly conserved and have probably been maintained by selective pressure, indicating that the functions of the gene products are required in cytoplasm and chloroplasts [16,25]. Both genes are highly conserved and have probably been maintained by selective pressure, indicating that the functions of the gene products are required in cytoplasm and chloroplasts [16,25]. Both genes are highly conserved and have probably been maintained by selective pressure, indicating that the functions of the gene products are required in cytoplasm and chloroplasts [16,25]. Both genes are highly conserved and have probably been maintained by selective pressure, indicating that the functions of the gene products are required in cytoplasm and chloroplasts [16,25].

Kinetic data suggested that both enzymes oxidize medium- to long-chain aliphatic aldehydes, with a preference for long-chain aldehydes. Notably, *K_m* values for the unsaturated C9-aldehyde trans-2-nonenal were in the same low range as the saturated nonanal. Enzymatic data indicated that substrate specificities for the two family 3 *Arabidopsis* ALDHs were determined by chain length rather than by saturation. This is in contrast with analyses from the enzymatic properties of human and rat ALDH3A1, which show that affinities are generally lower with unsaturated aldehydes than saturated aldehydes [41]. The kinetic parameters of *Arabidopsis* ALDH3H1 and ALDH3I1 were also different from the two family 2 maize mitochondrial ALDHs RF2A and RF2B. RF2A is unable to oxidize nonenal, but hexenal is a good substrate with a *K_m* value nearly three times lower than the *K_m* determined for the *Arabidopsis* enzymes. The other mitochondrial ALDH isoform RF2B is unable to use unsaturated aliphatic aldehydes [29]. Like animal family 3 ALDHs, ALDH3H1 and ALDH3I1 efficiently metabolized 4-hydroxynonenal, one of the α,β-unsaturated aldehydes that accumulates during lipid peroxidation [42]. *K_m* values of both *Arabidopsis* ALDHs for 4-hydroxynonenal were comparable with those of human and other animal ALDHs [41]. 4-Hydroxynonenal is also a very good substrate with a *K_m* in the low micromolar range for the maize mitochondrial RF2A ALDH [29]. In contrast, 4-hydroxynonenal is not a substrate for CptALDH from *Craterostigma plantagineum*, a close orthologue of ALDH3H1 and ALDH3I1 [16], and it is a poor substrate for alfalfa MsALR aldose/aldehyde reductase [43]. The substrate specificities for the ALDH3H1 and ALDH3I1 enzymes were similar except that the chloroplastic enzyme was slightly more efficient with dodecanal as substrate than the cytosolic enzyme, which reacted more efficiently with trans-2-nonenal than the chloroplastic isofrom. This may point to substrate specialization related to the two cellular compartments.

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**Figure 3** Enzymatic activities depend on the redox states of the ALDH proteins

ALDH activities of reduced (R) ALDH3H1 (A) and ALDH3I1 (B), after a 90 min incubation with 50 μM CuCl2 (O) and after re-reduction by incubation for 1 h with 10 mM DTT (RrD) or 10 mM reduced glutathione (RrG). The redox states of all tested fractions were analysed in parallel by non-reducing SDS/PAGE (10% gel). All values represent the means ± S.E.M. of three independent experiments. Asterisks denote statistically significant differences (*P* < 0.01, Student’s *t* test).

**Figure 4** ALDH inactivation during oxidation is correlated with loss of sulfhydryl groups

ALDH proteins were treated with 50 μM CuCl2 for up to 120 min. Enzyme activities (continuous lines) were determined and relative amounts of sulfhydryl groups were measured using DTNB (broken lines): ALDH3H1 (A) and ALDH3I1 (B).
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Figure 5 Activities of ALDH3H1, ALDH3I1 and their Cys-mutant proteins after oxidation and subsequent re-reduction

Enzyme activities were determined of the purified wild-type ALDH3H1 (A) and ALDH3I1 (B) and the corresponding Cys mutants (A and B) in different redox states. The bars represent the activities of the following samples: freshly purified proteins (R), proteins oxidized with 50 μM CuCl2 (O), and proteins oxidized and subsequently reduced by incubation with 10 mM DTT (RrD) or reduced glutathione (RrG). All values represent the means ± S.E.M. of at least three independent experiments. Asterisks denote statistically significant differences (P < 0.01, Student's t test). The mutated enzymes ALDH3H1Cys253Ser and ALDH3I1Cys316Ser are not shown, because these cysteine residues are part of the active centre and the corresponding mutants were inactive.

Coenzyme specificity

The coenzyme specificity of ALDH3H1 and ALDH3I1 was correlated with their subcellular localization. The cytosolic ALDH3H1 was unable to utilize NADP⁺ as a coenzyme, whereas the chloroplastic ALDH3I1 was able to use either NAD⁺ or NADP⁺. ALDH3H1 resembles the maize mitochondrial family 2 ALDHs RF2B and RF2A with regard to coenzyme specificity [29]. To our knowledge, this is the first report of a strictly NAD⁺-specific family 3 ALDH enzyme activity.

Crystal structures of several ALDHs indicate that they bind the coenzyme in a five stranded open α/β Rossmann fold [14,37]. Coenzyme specificity in ALDHs is determined by a web of different amino acids, but most importantly by a lysine residue that interacts with the adenine ribose or the 2'-phosphate of NAD⁺ and NADP⁺ respectively, and a glutamate residue that occupies a central position in the coenzyme binding site and co-ordinates the adenine ribose 2'- and 3'-hydroxyls of NAD⁺, while repelling the 2'-phosphate of the ribose of adenosine in NADP⁺ [37]. Thus space in the opposite side of the coenzyme binding cleft is required to keep the NADP⁺ molecule interacting in an active conformation. The NAD⁺-specific ALDH3H1 has an isoleucine residue instead of a valine in motif 4 (Figure 1A and Supplementary Figure S2). The isoleucine residue has a rigid and large hydrocarbon side chain, due to an additional methyl group compared with the equivalent valine occupying this position in ALDH3I1. A valine residue is invariant in this position in all family 3 ALDHs except for ALDH3H1 and ALDH3F1 (Figure 1A). Therefore it was tested whether the unusual isoleucine residue is the reason for the inability to use NADP⁺. The large β-branched hydrophobic side chain group oriented towards the coenzyme binding cleft restricts the available space for coenzyme binding. As a consequence it is difficult to accommodate the 2'-phosphate of the NADP⁺ molecule, whereas NAD⁺ interaction is not disturbed. This proposed scenario is supported by our observation that after replacing the isoleucine residue with valine, the cleft is wider and the distance from the valine to glutamic acid is lengthened to 9.23 Å, as determined by modelling (Figure 1). This may provide the additional space necessary to accommodate the 2'-phosphate group, thus resulting in a mutated enzyme that is able to use NADP⁺, but not affecting NAD⁺ binding (Table 2B).

Inserting a glycine residue in position 200 provided further support that the width of the coenzyme-binding cleft is critical. The removal of the side chain should enlarge the cleft to a width of 11.63 Å. This modification impaired NAD⁺ binding and simultaneously increased the affinity for NADP⁺ (Table 2B). The enlargement of the coenzyme-binding site resulted in a shift in
Figure 6  Redox sensitivities of ALDH3H1 and ALDH3I1 Cys-mutant proteins

Freshly purified ALDH3H1 (5 μg) (A) and ALDH3I1 (10 μg) (B) Cys-mutant proteins were obtained in the reduced form and were subsequently oxidized by incubation with 50 μM CuCl2 or 1 mM H2O2 at room temperature for 90 min. Then the redox state of each protein was analysed by non-reducing SDS/PAGE (10 % gel). Proteins were visualized using PageBlue® protein staining solution (Fermentas).

specificity from NAD+ to NADP+, documented by the relative \( K_{\text{mNAD}^+}/K_{\text{mNADP}^+} \) ratio. The replacement of the isoleucine residue by glycine provided the space required for accommodation of the 2’-phosphate group of the ribose of NADP+, but the larger space made the cleft too wide to facilitate a tight binding of NAD+. Thus the large isoleucine side chain is likely to force the adenine ribose of NAD+ to a proper distance from the enzyme surface inside the coenzyme-binding cleft. The present study elucidates the role of the amino acid residue positioned opposite the glutamic acid in the coenzyme-binding site as a determinant of nucleotide coenzyme specificity.

To further examine the influence of isoleucine residues in coenzyme binding, the valine in ALDH3I1 was replaced by isoleucine. This should create a smaller width across the coenzyme-binding cleft and nearly resemble the situation in ALDH3H1. Contrary to our expectations, the mutation of ALDH3I1 did not alter coenzyme affinities as binding of NADP+ was not weakened. The ALDH3I1Ile263Val mutant actually exhibited an increased affinity for NADP+, which demonstrates that not only the valine, but also additional amino acids, may influence coenzyme binding. Comparing the coenzyme affinities in ALDH3H1 and ALDH3I1 suggests that the coenzyme binding site environment is different in the two enzymes, which may explain the result obtained for the ALDH3I1Ile263Val mutant.

Redox state

The redox and oligomeric states also determined the enzymatic activities of the ALDH proteins. Under oxidizing conditions, ALDH3H1 as well as ALDH3I1 were susceptible to thiol oxidation, which led to decreased activities. Oxidative inactivation and recovery after reduction allow the conclusion that thiol regulation and not unspecific oxidation takes place. Thiol regulation involves at least one redox-sensitive residue, which is critical for the disulfide-bond-mediated dimerization, in addition to other oxidatively modified thiol groups. ALDH3H1Cys253Ser showed enzyme activity and the deactivation rate upon oxidation was very similar to the non-mutated enzyme. However, the Cys253Ser mutation caused a complete inactivation, which supports the crucial role of this cysteine residue responsible for a nucleophilic attack on the carbonyl carbon of the aldehyde substrate leading to the enzyme-linked thiohemiacetal intermediate during catalysis [38–40]. Mutation of Cys45, which is not situated in the vicinity of the catalytic site (Figure 8), caused a loss of more than 75% of the initial activity of the wild-type enzyme and abolished intermolecular disulfide bond formation. This suggests that Cys45 is the redox-responsive residue required to form intermolecular disulfide bonds under oxidizing conditions. This cysteine residue may act as a sensor and release active enzyme depending on the redox environment.

ALDH3H1 contains six cysteine residues (Supplementary Figure S2 and Supplementary Table S1). As the Cys316Ser mutant was inactive, this proves that Cys316 is essential for the catalytic activity occupying the same position as Cys253 in ALDH3H1. The mutation of Cys114 abolished dimerization under oxidizing conditions and caused a decrease of enzymatic activity, which suggests that Cys114 is responsible for intermolecular disulfide bridge formation in homodimers. Cysteine residues mediating
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Figure 7 Regeneration of monomers from ALDH3H1 and ALDH3I1 Cys mutants after treatment with different concentrations of DTT

Freshly purified proteins were oxidized by incubation with CuCl₂ and then treated with different concentrations of DTT for 1 h to re-reduce the proteins. The protein samples [5 μg for ALDH3H1 (A) and 10 μg for ALDH3I1 (B)] were separated by non-reducing SDS/PAGE (10 %) and stained with PageBlue® protein staining solution (Fermentas). Monomers and dimers are shown.

redox-dependent dimerization are located in the N-terminal domains of both ALDH isoforms.

Modelling of the three-dimensional structure of Arabidopsis ALDH3H1 and ALDH3I1 was performed using the crystallized ALDH3A1 structure from rat as template [14]. Comparison of the structure of the rat ALDH3A1 and the predicted models of ALDH3H1 and ALDH3I1 revealed that the region corresponding to the domain harbouring the cysteine residues, which form the intermolecular disulfide bridge between two subunits, is located on opposite sides of the hydrogen-linked homodimer (Figure 8). Redox-sensitive cysteine residues appear to be located in the subunit interface, in a half-buried position. Under reducing conditions, the sulfhydryl side chains of Cys45 and Cys114 residues in ALDH3H1 and ALDH3I1 respectively may spatially not be close enough to establish a disulfide bridge in the hydrogen-bond-stabilized homodimer interface. Therefore oxidation should trigger conformational changes that alter the surface charges and the hydrophobicity, thereby bringing the thiol group into a more exposed and closer position favourable for forming intermolecular disulfide bonds with the other subunit. Those changes may also alter the enzyme shape into a low-activity conformation that facilitates additional intermolecular interactions allowing the formation of multimers (Figures 2A and 2C).

Densitometric analysis of protein patterns of oxidized fractions showed that the ratio between dimers and monomers does not correlate well with the observed loss of activity (Supplementary Table S3). Thus one can assume that other cysteine residues were also affected by oxidation. The detection of thiol sulfinate and thiol sulfonate products in MALDI–TOF-MS

Figure 8 Ribbon and stick diagrams of the predicted structures of A. thaliana ALDH3H1 and ALDH3I1 monomeric subunits

Molecules are coloured in grey. Green asterisks in the ribbon diagrams denote the central helices of the coenzyme-binding Rossmann-fold domain. Catalytic cysteine residues are depicted in yellow, redox-sensitive cysteines in red and other cysteines in cyan. The insets of the stick diagrams show the location of the redox-sensitive cysteines in a half-buried position of the subunit interfaces of ALDH3H1 (A) and ALDH3I1 (B). The predicted structures of ALDH3H1 and ALDH3I1 were built using the Web-based modelling server SWISS-MODEL [35] and the solved crystal structure of the R. norvegicus ALDH3A1 as template [14]. Obtained structures were rendered using PyMol. Regions highlighted in orange in the ribbon diagrams indicate helix αD and sheets β12 as well as β13 involved in hydrogen bond-mediated homodimerization in the functional native homologous ALDH3A1 protein [14]. The position of the hexa-His-tag located in the N-terminus is not indicated, but is represented in the generated models (♣).
(matrix-assisted laser-desorption ionization–time-of-flight-MS) analysis of monomers in the oxidized fraction of ALDH3H1 suggests this assumption (results not shown). It appears that a probable basis for the reversible inactivation of both enzymes under oxidizing conditions is due to the oxidation of their cysteine residues. Oxidation of ALDHs was always performed by incubation with $50 \mu$M CuCl$_2$. Thus it is possible that copper, or one of its catalysed oxidation products, reacts with thiols before the generation of disulfides. This might result in an irreversible formation of oxidized thiol products, i.e. sulfenic acid or more highly oxidized forms such as sulfonic and sulfonic acid or oxidation of methionines to methionine sulfoxides, which explains why enzymatic activity could not be completely restored after re-reduction.

Redox sensitivity seems to be a more general feature of dehydrogenase enzymes. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) is susceptible to oxidation. Human GAPDH undergoes disulfide bond formation and loss of thiol groups, which leads to a reduction in its enzymatic activity [44]. Similar to oxidized ALDH3H1 and ALDH3I1, GAPDH activity was not fully restored when the oxidized form was treated with reducing agents [45]. Another example is the Arabidopsis cytMDH (cytosolic malate dehydrogenase), which lost activity after oxidation and subsequent homodimer formation; also activities could only be partially restored [46]. After nitroglycerin-mediated S-nitrosylation, a weaker reactivation was reported for mitochondrial ALDH2 [47]. This suggests that reversible thiol oxidation may be an important mechanism for the post-translational control of plant ALDHs.

The inactivation of ALDHs after dimerization under oxidizing conditions was unexpected, since previous in vivo experiments demonstrated that ALDH3H1 and ALDH3I1 can protect against oxidative stress [25]. Experiments with transgenic plants suggested that both enzymes should be efficient under oxidative stress conditions and able to oxidize toxic lipid peroxidation-derived products. Therefore it seems necessary to maintain aldehyde-detoxifying activities even in an oxidizing environment. This raises the question whether ALDHs interact with another protein partner to avoid the redox modification. It is conceivable that activity could be maintained by involving a physiological reductant such as thioredoxin protecting the N-terminal cysteines [48]. Formation of heterodisulfides between thioredoxin or glutaredoxins and the redox-sensitive cysteine residues could prevent protein oxidation and loss of activity via homodimerization. This hypothesis seems to be plausible, because it was shown that the redox-sensitive dehydrogenases cytMDH and GAPDH are targets of cysteolic thioredoxins [49]. The reduced form of thioredoxin h1 efficiently reduces and reactivates cytMDH in vitro after oxidation [46]. This assumption is supported by reports that have identified plant ALDHs as potential target proteins for thioredoxin and glutaredoxin [50].

Thioredoxin-dependent activation of redox-sensitive chloroplastic enzymes has been well documented in vascular plants. Chloroplastic NADP-MDH (NADP-dependent malate dehydrogenase) of sorghum is targeted by thioredoxin and it is activated via the reduction of its intramolecular disulfide bridges [51]. Protein–protein interactions involving thioredoxins to modulate dehydrogenase activity in chloroplasts under oxidative stress conditions are an ancient feature. The NADP-MDH from the unicellular green alga *Chlamydomonas reinhardtii* is also redox-regulated and activated by the thioredoxin f1 [52]. Thioredoxin regulation has also been reported for chloroplastic GAPDH [53]. We are therefore initiating research to assess the role(s) that thioredoxin and protein–protein interactions may play in the control of ALDH3H1 and ALDH3I1 during oxidative stress.

**REFERENCES**


**AUTHOR CONTRIBUTION**

Naim Stiti was responsible for most of the experimental work and made a major contribution to the writing of the manuscript. Isaac Adewale was responsible for carrying out a large part of the enzymatic activity tests and corresponding data analysis. Jan Petersen was responsible for discovery of the dimer formation under different redox conditions. Dorothea Bartels was responsible for supervision and guidance of the experimental work, data analysis and major corrections of the manuscript prior to publication. Hans-Hubert Kirch was responsible for supervision and guidance of the experimental work, data analysis and writing of the manuscript.

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SUPPLEMENTARY ONLINE DATA
Engineering the nucleotide coenzyme specificity and sulfhydryl redox sensitivity of two stress-responsive aldehyde dehydrogenase isoenzymes of Arabidopsis thaliana

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Figure S1 Typical protein elution profiles, protein immunoblot analysis and pH-optima of recombinant ALDH3H1 and ALDH3I1 enzymes after affinity chromatography

Samples of 5 μl of each collected fraction of affinity-purified ALDH3H1 and ALDH3I1 proteins were separated by SDS/PAGE (10 % gel) and stained with colloidal Coomassie Blue (A and C), or transferred to nitrocellulose membranes and analysed with anti-ALDH antisera (1:5000) (B and D). Elution profile of ALDH3H1 (E) and ALDH3I1 (F) from the His-affinity column (continuous lines) and corresponding enzymatic activities of each fraction (broken lines). Protein concentrations were measured using the Bradford assay (Bio-Rad). ALDH activities were determined using the freshly extracted fractions with hexanal as substrate as described in Experimental section in the main text. (G) pH-optima were determined for purified ALDH3H1 and ALDH3I1 enzymes with 0.1 M sodium phosphate (grey squares and triangles) for the pH range 6.0 to 8.0, and with 0.1 M sodium pyrophosphate (black squares and triangles) for the pH range 8.5 to 10.0. Enzyme assays were conducted at room temperature with 1 mM hexanal and 1.5 mM NAD⁺.

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Figure S2  Amino acid sequence alignment of selected family 3 ALDHs from Arabidopsis, Craterostigma and rat

UniProt accession numbers are listed in brackets. Amino acid sequences of A. thaliana (ARATH) ALDH3F1, ALDH3H1 and ALDH3I1 (Q70E96, Q70DU8 and Q8W033 respectively), C. plantagineum (CRAPL) Cp-ALDH (Q8VXQ2) and ALDH3A1 from rat (P11883) were aligned using the AlignX program (Invitrogen, Vector NTI-Suite v. 10) and edited with GeneDoc [1]. Identical amino acids are shaded in black. Amino acids of the plastid targeting sequence of ALDH3I1 are shown in light blue. Conserved sequence motifs involved in catalysis (motifs 1, 5 and 6) and coenzyme binding (motifs 2 and 4) are boxed [2]. Cysteine residues are highlighted in red. Residues that are important for NAD+/NADP+ preference in ALDHs are highlighted in blue (lysine and glycine) and green (glutamic acid) [3–5]. Conserved amino acids that may be important for coenzyme binding in ALDH3H1 and ALDH3I1 are indicated in green (valine) and in pink (isoleucine) (see the main text for details). Amino acid residue positions are indicated by numbers on the right hand side for each individual protein.
Table S1  Generated ALDH3H1 and ALDH3I1 mutants

The Table lists the generated mutants and the positions of the mutated amino acids in the native and the recombinant proteins (the positions are not identical because of the cloning procedure) and the primers used to generate the mutated proteins. Bold and underlined letters in oligonucleotide sequences refer to substituted nucleotides.

<table>
<thead>
<tr>
<th>Mutated amino acid positions</th>
<th>Oligonucleotide used to create the desired mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALDH mutants</td>
<td>Native enzyme Recombinant enzyme Name Sequence (5′ to 3′)</td>
</tr>
<tr>
<td>Coenzyme affinity studies</td>
<td></td>
</tr>
<tr>
<td>ALDH3H1</td>
<td>Ile200Val 200 229</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>ALDH3H1</td>
<td>Ile200Gly 200 229</td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Redox regulation and dimer formation studies</td>
<td></td>
</tr>
<tr>
<td>ALDH3H1</td>
<td>Cys247Ser 247 276</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>ALDH3H1</td>
<td>Cys253Ser 253 282</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>ALDH3I1</td>
<td>Cys114Ser 114 97</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>ALDH3I1</td>
<td>Cys142Ser 142 125</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>ALDH3I1</td>
<td>Cys286Ser 286 269</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>ALDH3I1</td>
<td>Cys310Ser 310 293</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>ALDH3I1</td>
<td>Cys316Ser 316 299</td>
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Table S2  Overall yields of ALDH3H1 and ALDH3I1 purified recombinant enzymes

<table>
<thead>
<tr>
<th>Protein concentration (μg/μl)</th>
<th>Total protein yield (mg)</th>
<th>Total units (μmol NADH x min⁻¹ x 250 μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein concentration (μg/μl)</td>
<td>Total protein yield (mg)</td>
<td>Total units (μmol NADH x min⁻¹ x 250 μl)</td>
</tr>
<tr>
<td>I</td>
<td>II</td>
<td>III</td>
</tr>
<tr>
<td>ALDH3H1 (WT)</td>
<td>1.24</td>
<td>1.19</td>
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<tr>
<td>ALDH3H1Cys45Ser</td>
<td>0.22</td>
<td>0.31</td>
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<tr>
<td>ALDH3H1Cys247Ser</td>
<td>0.70</td>
<td>0.73</td>
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<tr>
<td>ALDH3H1Cys253Ser</td>
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<tr>
<td>ALDH3H1Ile200Val</td>
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<tr>
<td>ALDH3H1Ile200Gly</td>
<td>1.14</td>
<td>0.93</td>
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<tr>
<td>ALDH3I1 (WT)</td>
<td>0.67</td>
<td>0.56</td>
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<tr>
<td>ALDH3I1Cys114Ser</td>
<td>0.30</td>
<td>0.38</td>
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<td>ALDH3I1Cys142Ser</td>
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<tr>
<td>ALDH3I1Cys286Ser</td>
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<tr>
<td>ALDH3I1Cys310Ser</td>
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<tr>
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<td>ALDH3I1Val263Ile</td>
<td>0.83</td>
<td>0.76</td>
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Table S3  Amounts of ALDH monomers compared with dimers in comparison to enzymatic activities under different redox conditions

Relative amounts of ALDH monomers compared with disulfide-linked dimers and their correlation with enzymatic activity in different redox states. Densitometric analyses of the non-reducing SDS/PAGE gels were performed using ImageJ software (http://rsbweb.nih.gov/ij/). Results are mean values ± S.E. from three independent experiments.

<table>
<thead>
<tr>
<th>Redox state</th>
<th>ALDH3H1</th>
<th></th>
<th>ALDH3H1</th>
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<tbody>
<tr>
<td></td>
<td>Monomers (%)</td>
<td>Dimers (%)</td>
<td>Activity (%)</td>
<td>Monomers (%)</td>
</tr>
<tr>
<td>Reduced</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Oxidized</td>
<td>66.9 ± 4.3</td>
<td>33.1 ± 2.3</td>
<td>23.8 ± 1</td>
<td>87.6 ± 6</td>
</tr>
<tr>
<td>Re-reduced (10 mM GSH)</td>
<td>85.6 ± 5.6</td>
<td>14.4 ± 1.6</td>
<td>44 ± 2.3</td>
<td>93.6 ± 5.1</td>
</tr>
<tr>
<td>Re-reduced (10 mM DTT)</td>
<td>86.2 ± 5.1</td>
<td>13.8 ± 2</td>
<td>82.9 ± 4.4</td>
<td>96.1 ± 6.1</td>
</tr>
</tbody>
</table>

REFERENCES


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