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Amino Acid Residues Critical for the Specificity for Betaine Aldehyde of the Plant ALDH10 Isoenzyme Involved in the Synthesis of Glycine Betaine

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ABSTRACT

Plant ALDH10 enzymes catalyze the oxidation of ω-primary or ω-quaternary aminoaldehydes but, intriguingly, only some of them, such as the Spinacia oleracea betaine aldehyde dehydrogenase (SoBADH), efficiently oxidize betaine aldehyde (BAL) forming the osmoprotectant glycine betaine (GB), which confers tolerance to osmotic stress. The crystal structure of SoBADH reported here shows Tyr-160, Trp-167, Trp-285, and Trp-456 in an arrangement suitable for cation-π interactions with the trimethylammonium group of BAL. Mutation of these residues to alanine resulted in significant $K_m$(BAL) increases and $V_{max}/K_m$(BAL) decreases, particularly in the Y160A mutant. Tyr-160 and Trp-456, strictly conserved in plant ALDH10s, form a pocket where the bulky trimethylammonium group binds. This space is reduced in ALDH10s with low BADH activity because an isoleucine pushes the tryptophan against the tyrosine. Those with high BADH activity instead have alanine (Ala-441 in SoBADH) or cysteine, which allow enough room for binding of BAL. Accordingly, the mutation A441I decreased $V_{max}/K_m$(BAL) of SoBADH ~200-times while the mutation A441C had no effect. The kinetics with other ω-aminoaldehydes were not affected in the A441I or A441C mutants, demonstrating that the existence of an isoleucine in the second sphere of interaction of the aldehyde is critical for discriminating against BAL in some plant ALDH10s. A survey of the known sequences indicate that plants have two ALDH10 isoenzymes; those known to be GB-accumulators have a high-BAL-affinity isoenzyme with alanine or cysteine in this critical position, while non-GB-accumulators have low-BAL-affinity isoenzymes containing isoleucine. Therefore, BADH activity appears to restrict GB synthesis in non-GB-accumulator plants.
INTRODUCTION

Osmotic stress caused by drought, salinity or low temperatures is a major limitation of agricultural production. Some plants synthesize and accumulate glycine betaine (GB)—the most efficient osmoprotector known (Courtenay et al., 2000)—when subjected to osmotic stress (Yancey et al., 1982; Hanson and Wyse 1982; Weretilnyk et al., 1989; Valenzuela-Soto and Muñoz-Clares, 1994). It is generally accepted that GB is synthesized in the chloroplast stroma, as it is in spinach (Hanson et al., 1985), by a two-step oxidation of choline: first the alcohol group of choline is oxidized to the aldehyde group of betaine aldehyde (BAL) in a reaction catalyzed by choline monooxygenase (E.C.1.14.15.7, CMO), an enzyme unique to plants (Burnet et al., 1995); then the aldehyde group of BAL is oxidized to the acid group of GB in a reaction catalyzed by plant betaine aldehyde dehydrogenase (betaine aldehyde: NAD(P)+ oxidoreductase, E.C. 1.2.1.8, BADH) (Hanson et al., 1985; Weretilnyk and Hanson, 1989; Arakawa et al., 1987; Valenzuela-Soto and Muñoz-Clares, 1994; Burnet et al., 1995; Hibino et al., 2001; Nakamura et al., 2001; Fujiwara et al., 2008; Kopěcný et al., 2011), an enzyme that belongs to the aldehyde dehydrogenase (ALDH) family 10 (ALDH10) (Vasiliou et al., 1999). Engineering the synthesis of GB in crops that naturally lack this ability has been a biotechnological goal for improving tolerance to osmotic stress (McNeil et al., 1999; Rontein et al., 2002; Waditee et al., 2007). The several attempts made so far have had limited success, stressing the need for a better understanding of the structural and functional properties of the enzymes involved in the GB biosynthetic pathway.

Most biochemically characterized plant ALDH10s appear to be ω-aminoaldehyde dehydrogenases (AMADHs) that can oxidize small aldehydes possessing an ω-primary amine group, such as 3-aminopropionaldehyde (APAL) and 4-aminobutyraldehyde (ABAL) (Vojtěchová et al., 1997; Trossat et al, 1997; Šebela et al, 2000; Livingstone et al., 2003; Oishi and Ebina, 2005; Fujiwara et al., 2008; Bradbury et al., 2008), or an ω-quaternary amino (trimethylammonium) group, as in BAL and 4-trimethylaminobutyraldehyde (TMABAL) (Brauner et al., 2003; Fujiwara et al., 2008). In addition, unsurprisingly given the structural similarity between the trimethylammonium and the dimethylsulfonium groups, some plant BADHs can also use as substrate 3-dimethylsulfoinopropionaldehyde to produce the osmoprotectant 3-
Because of their relatively broad specificity, the plant ALDH10 enzymes may be involved not only in synthesis of these two osmoprotectants but also in the synthesis of others such as β-alanine betaine (Rathinasabaphathi et al., 2000) and 4-aminobutyric acid, (Bouché and Fromm, 2004), as well as in polyamines catabolism and in synthesis of carnitine (Fig. 1). To date, there are no other known plant ALDHs with AMADH or BADH activities other than the ALDH10 enzymes. With the exception of the two BADH isoenzymes from the mangrove plant *Avicennia marina* (Hibino et al., 2001), plant ALDH10s oxidize ω-primary-aminoaldehydes with similar, or even higher, efficiency than they oxidize BAL, but some of these enzymes exhibit a very low activity with BAL (Šebela et al., 2000; Livingstone et al., 2003; Fujiwara et al, 2008; Bradbury et al., 2008) for as yet unknown reasons. On the basis of their affinity and activity with BAL, it has been proposed that plant BADHs constitute two subfamilies: true BADHs and high BADH homology aminoaldehyde dehydrogenases (Fitzgerald et al., 2009).

Neither how some ALDH10 enzymes discriminate against BAL, nor the structural bases for BAL binding are known. Here, for the first time, we present the crystal structure of a chloroplastic ALDH10 with high BADH activity, the one from spinach (*Spinacia oleracea*) (SoBADH), and report the results of site-directed mutagenesis of the residues involved in binding of BAL, as well as of the critical residue involved in discriminating BAL from other aminoaldehydes in those plant ALDH10s with poor BADH activity.

**RESULTS**

**Overall description of the SoBADH three-dimensional structure**

The structure of SoBADH in complex with NAD⁺ was determined at 2.3 Å resolution. The crystal belongs to the P1 space group and contains four subunits in the asymmetric unit describing two dimers, each of which corresponds to the biological unit of the enzyme (Valenzuela-Soto and Muñoz-Clares, 1994). The final model has an *R*<sub>work</sub> value of 21.3 % and an *R*<sub>free</sub> value of 24.6 %. All data collection and refinement statistics are summarized in Supplemental Table S1. The folding (Supplemental Fig. S1) and topology (Supplemental Fig. S2) are very similar to those of every ALDH of known three-dimensional structure. The two K⁺-binding sites, one
intra-subunit, the other inter-subunit, which were previously found in the tetrameric BADH from *Pseudomonas aeruginosa* (González-Segura et al., 2009), also occur in the dimeric SoBADH.

**Role of aromatic residues in the betaine aldehyde binding-site of SoBADH**

An inspection of the SoBADH crystal structure revealed four aromatic residues, Tyr-160, Trp-167, Trp-285, and Trp-456 in an arrangement suitable for the binding of the trimethylammonium group of BAL through cation-π interactions (Fig. 2A). As a first step to evaluate whether these residues play a role in substrate recognition and enzyme specificity, we constructed an energy-minimized model of the SoBADH active site with BAL productively bound, so that its carbonyl oxygen is inside the oxyanion hole (Muñoz-Clares et al., 2010) accepting two hydrogen bonds, one from the side-chain amide nitrogen of Asn-159 and another from the main-chain nitrogen of the catalytic cysteine (Cys-291, SoBADH numbering), and its carbonyl carbon in a position suitable for accepting the nucleophilic attack of the catalytic cysteine. In the model, the trimethylammonium group is surrounded by the four aromatic residues at distances in the range of 3.2 to 4.2 Å, consistent with their involvement in BAL binding (Fig. 2B). The tunnel through which the aldehyde enters the active site has an irregular shape, widening in the vicinity of the catalytic cysteine (Fig. 2C). The molecular electrostatic potential of the surface of this tunnel is clearly negative, mainly due to glutamate and aspartate residues near the tunnel entrance, and to the aromatic rings of the four residues mentioned above, located where the trimethylammonium group binds (Fig. 2C). The side-chain aromatic electrons contribute to the binding of the positively charged trimethylammonium group of BAL through cation-π interactions.

To determine the relative contribution of these four aromatic residues to the binding of BAL, we individually mutated each of them to alanine, so to exclude the possibility for π-cation stabilization of the BAL trimethylammonium group. The kinetics of the pure mutant enzymes were studied with BAL, APAL, ABAL, and TMABAL as substrates. Steady-state kinetics studies were carried out at pH 8.0, which is close to the physiological intrachloroplast pH value in spinach under light conditions (7.88; Werdan and Heldt, 1972), and at fixed 0.2 mM NAD⁺, which is the concentration estimated to exist in the stroma of spinach chloroplast (0.19; Heineke et al., 1991). This NAD⁺ concentration was found to be saturating or near saturating for
the wild-type and the mutant enzymes, in experiments in which the concentration of NAD$^+$ was varied at a fixed concentration of BAL (a high but non-inhibitory concentration which was at least 4-times the $K_m$ value of each enzyme) (Supplemental Table S2).

The wild-type SoBADH exhibited kinetic parameters for BAL, APAL and ABAL (Table I and Fig. 2D) similar to those reported earlier for this enzyme (Incharoensakdi et al., 2000). The kinetics of TMABAL were comparable to those of APAL and ABAL. The $V_{\text{max}}$ and $K_m$ values determined using BAL as substrate were between 4- and 9-times and between 12- and 26-times higher, respectively, than the values obtained using the other ω-aminoaldehydes, whose tighter binding to the enzyme appears to correlate with a slower catalysis. This results in a higher (between 1.2- and 4.3-times) catalytic efficiency (measured as $V_{\text{max}}/K_m$) for the other ω-aminoaldehydes tested than for BAL (Table I and Fig. 2D). The mutant enzymes exhibited significantly increased $K_m(BAL)$ and decreased $V_{\text{max}}/K_m(BAL)$ values, particularly the Y160A mutant, which had 140-times higher $K_m(BAL)$ and a 550-times lower $V_{\text{max}}/K_m(BAL)$ than the wild-type SoBADH (Table I and Fig. 2D), indicating that the tyrosine aromatic ring is of the utmost importance for binding of the aldehyde. On the basis of the observed changes in $K_m(BAL)$, Trp-285 also appears to be very important for the productive BAL binding, followed by Trp-167 and last by Trp-456. Although in our energy-minimized model Trp-285 is the farthest from the methyl groups of BAL, the significant effect of its mutation may be in part due to the loss of the van der Waals interactions of its indole ring carbon atoms CH2 and CZ3 with the phenol oxygen of Tyr-160. These interactions contribute to maintain the position of Tyr-160 that allows binding of the trimethylammonium group (see below). The mutant enzymes exhibited decreased $V_{\text{max}}$ values, but to a lesser extent than the increases in $K_m$ values. The exception was the W167A mutant, which had a greater effect on $V_{\text{max}}$ than in $K_m$. Interestingly, the mutation of the four aromatic residues had a much lesser effect on the kinetics of SoBADH with APAL, ABAL and TMABAL than on those of BAL (Table I and Fig. 2D). The mutant enzymes W167A, W285A and W456A exhibited small changes in $K_m$ for APAL, ABAL and TMABAL compared with the wild-type enzyme, indicating that their affinity for these aldehydes has not been importantly affected and, therefore, that these aromatic residues do not contribute to their binding. The $V_{\text{max}}$ values determined using these aldehydes as
substrates were not importantly affected by the mutation of these three aromatic residues. Consequently, neither were the $V_{\text{max}}/K_m$ values. The Y160A mutant, however, exhibited an 80-times increase in the $K_m$ value for APAL, and around 20-times increases in the $K_m$ values for ABAL and TMABAL with respect to the wild-type SoBADH, resulting in importantly decreased $V_{\text{max}}/K_m$ values. Multiple alignments of the known plant ALDH10 amino acid sequences indicated that Tyr-160, Trp-167 and Trp-456 are strictly conserved residues in these enzymes, whereas Trp-285 is a phenylalanine or an alanine in some of them. On the basis of our results, it could be speculated that those enzymes with an alanine in the position equivalent to Trp-285 in SoBADH would use APAL, ABAL and TMABAL as substrates preferentially to BAL.

**Residues involved in discriminating against binding of BAL in plant ALDH10 enzymes**

To date, there are only three plant ALDH10 enzymes whose three-dimensional structure is known: the SoBADH reported here (PDB code 4A0M) and two isoenzymes from *Pisum sativum* (PsAMADH1 and PsAMADH2, PDB codes 3IWK and 3IWJ, respectively) (Tylichová et al., 2010). As none of the pea enzymes can use BAL as substrate (Šebela et al., 2000; Tylichová et al., 2010) whereas the spinach enzyme uses this aldehyde very efficiently, we compared the active sites of SoBADH and PsAMADH2 to find out the structural reasons for this important difference between them. We choose PsAMADH2 for this comparison because it has a tryptophan residue in the position of Trp-285 of SoBADH whereas PsAMADH1 has a phenylalanine. The superposition of the aldehyde-binding sites of the two enzymes shows that every residue lining the aldehyde entrance tunnel has a very similar conformation in both of them, with the exception of the side-chain of the tryptophan residue equivalent to Trp-456 (Trp-459 in PsAMADH2), which in the pea enzyme is closer to the phenol group of the side-chain of the tyrosine residue equivalent to Tyr-160 (Tyr-163 in PsAMADH2) than in the spinach enzyme. This results in a narrower cavity in PsAMADH2 than in SoBADH at the place where the bulky trimethylammonium group of the BAL should be accommodated (Figs. 3A and B). Energy-minimized models of the productively bound BAL molecule indicated that the trimethylammonium group can be bound in SoBADH but that it clashes with the
tryptophan residue in PsAMADH2 (Fig. 3C). The reason for this is the different position of the tryptophan side-chain in PsAMADH2, which is pushed towards the phenol group of the tyrosine by the side-chain of a non-active site residue—an isoleucine (Ile-444, PsAMADH2 numbering)—, which is behind and in close contact with the indole ring, at van der Waals distance (3.6 Å). Instead of Ile-444 SoBADH has Ala-441, whose much smaller side-chain allows Trp-456 to be at a distance from Tyr-160 sufficient for accommodating the trimethylammonium group of BAL (Fig. 3C). To investigate whether this difference in a residue in the second sphere of interaction of the aldehyde could account for the differences in BAL specificity between the spinach and pea enzymes, we individually mutated Ala-441 in SoBADH for an isoleucine. As expected, the mutant A441I enzyme showed a greatly reduced affinity for BAL, indicated by a $K_m$BAL value 23-times higher than that of the wild-type enzyme. It also exhibited a ~7-times lower $V_{max}$, which result in a decrease in the catalytic efficiency of the mutant A441I with BAL as substrate, $V_{max}/K_m$(BAL), of around 160-times. On the contrary, the $K_m$ and $V_{max}$ values for the other ω-aminoaldehydes were very similar to those of the wild-type enzyme (Table II and Figs. 3D and E). These results confirm our hypothesis of the critical importance of the side-chain of the residue behind the indole ring of the tryptophan residue equivalent to Trp-456 for plant ALDH10s to discriminate against BAL.

The only other plant ALDH10 that so far has been found to have a very poor affinity for BAL is the barley BBD1 (Fujiwara et al., 2008), an enzyme that also has an isoleucine in this position. Interestingly, barley has another ALDH10 isoenzyme with a high affinity for BAL, called BBD2, which possesses a cysteine residue in the position of Ala-441 of SoBADH (Cys-439, BBD2 numbering). We anticipated that in this enzyme the steric impediment for the binding of the trimethylammonium group of BAL does not occur because the small size of the cysteine side-chain. To prove this, we constructed the A441C mutant and confirmed that this change did not affect the $V_{max}$ and had a slight negative effect on $K_m$(BAL), which was increased 1.7-times compared with the wild-type value (Table II and Figs. 3D and E). These findings give additional support to our proposal of the critical importance of a small residue behind the indole group of the tryptophan residue for allowing BAL binding. The saturation kinetics of APAL, ABAL and TMABAL were not affected in the A441C mutant, as it was also expected.
DISCUSSION

The positive charge of the quaternary nitrogen of the trimethylammonium group of BAL suggests that negatively charged active-site residues should be involved in conferring substrate specificity to plant BADHs, by analogy with other enzymes that binds this group (Quaye et al., 2008). In a first study with SoBADH, Glu-103, which is strictly conserved in the known plant ALDH10 enzymes, was thought to be this residue; when changed to glutamine, however, there were no change in the kinetics with BAL as substrate and only a small negative effect on those with APAL and ABAL (Incharoensakdi et al., 2000). The crystal structure of the spinach enzyme reported here explains these results: the side-chain carboxylic group of Glu-103 is far from the aldehyde tunnel (Supplemental Fig. S3). ALDH10 enzymes also have two conserved aspartates (Asp-107 and Asp-110, SoBADH numbering), whose carboxyl groups are exposed, or partially exposed in the case of Asp-107, to the solvent filling the aldehyde tunnel. The energy-minimized model of the productively bound BAL indicates that these carboxyl groups are too far away from the trimethylammonium group to directly interact with it. While this paper was in preparation, Kopečný et al. (2011) reported marked decreases in the affinity for APAL and ABAL of PsAMADH2 mutants in which these two aspartates were changed to alanines. Our energy-minimized models with these aminoaldehydes productively bound (not shown) showed that Asp-107 and Asp-110 are more than 7.5 Å away from the amino group of ABAL and APAL. Since the carboxyl of Asp-110 is relatively close to that of Asp-107, and therefore may influence its position, and the carboxyl group of Asp-107 is at appropriate distance from Tyr-160 to electrostatically interact with the aromatic ring, the observed negative effects of the mutations D107A and D110A in PsAMADH2 may be, at least in part, due to the loss of this latter interaction, which may be relevant for the correct positioning of the tyrosyl residue equivalent to Tyr-160, a residue which is critical for the binding of the aldehydes (see below).

On the other hand, the trimethylammonium group of choline or GB has been shown to bind to proteins mainly through cation-π interactions with aromatic residues (Holtmann et al., 2004; Horn et al., 2006). The crystal structure of SoBADH showed four aromatic residues, Tyr-160, Trp-167, Trp-285, and Trp-456 in an arrangement
suitable for cation-π interactions with the trimethylammonium group of BAL (Fig. 2). The residue equivalent to Trp-167 was suspected to participate in binding BAL in the BADH from cod liver (Johansson et al., 1998), which is an ALDH9 not an ALDH10 enzyme (Vasiliou et al., 1999), but this possibility was discarded on the basis that this residue is conserved in several other ALDHs that are not specific for BAL. The architecture of the BAL-binding site in SoBADH is similar but not exactly the same to that of the GB-binding site in the GB transporter from Bacillus subtilis (Horn et al., 2006; PDB code 2B4L). While in the transporter there are only three tryptophanyl residues forming a prism that perfectly accommodates and binds the trimethylammonium group in a fixed position, in SoBADH there are four aromatic residues that could interact with the trimethylammonium group even if this group adopts different positions inside the active site. This allows a certain degree of flexibility in the binding of BAL that is needed for catalysis. The chemical mechanism of the ALDH catalyzed reactions involves intermediates covalently attached to the catalytic cysteine in which the trigonal carbonyl carbon of the aldehyde substrate changes to a tetrahedral carbon. First, the nucleophilic attack of the catalytic cysteine on the carbonyl carbon results in formation of a tetrahedral thiohemiacetal intermediate; second, the oxidation of this intermediate by the transfer of an hydride to NAD(P)⁺ forms a trigonal thioester intermediate; and then the nucleophilic attack of the hydrolytic water molecule to the thioester forms a new tetraheedral intermediate which collapses into the trigonal acid product of the reaction. The oxygen atom remains in the oxyanion hole but the rest of the molecule moves inside the active site as the geometry of the carbonyl carbon repeatedly changes (Muñoz-Clares et al., 2010). If the trimethylammonium group were bound in a fixed position, these movements would be hindered and catalysis negatively affected.

The kinetics of the SoBADH mutant enzymes in which the four aromatic residues were separately changed were consistent with the involvement of these residues in binding of the trimethylammonium group. Our results indicate that Tyr-160 is an important residue for binding the shortest ω-aminoaldehydes BAL and APAL, particularly for BAL, but also for binding of ABAL and TMABAL in spite of their amino group being farther from the aromatic ring. Interestingly, the $K_m$ for propionaldehyde, which lacks the amino group, is increased 50-times in the SoBADH
Y160A mutant (results not shown). The energy-minimized model constructed by us suggests that the carbonyl oxygen of the aldehyde when bound into the oxyanion hole is at van der Waals distance from the CE1 carbon of the phenol ring and in the same plane. In this position, the carbonyl oxygen can electrostatically interact with the positive pole of the aromatic ring quadrupole. In this way, Tyr-160 may be involved in the binding of any aldehyde. Most known ALDH sequences have a tyrosine or a phenylalanine in this position (Julián-Sánchez et al., 2007), supporting the relevance of the aromatic ring for binding of the aldehyde group, and probably also for its correct positioning for the following catalytic steps.

Our kinetic results with the mutant enzymes indicate that the binding of BAL to SoBADH depends on the interactions with the four aromatic residues much more than the binding of APAL, ABAL and TMABAL, i.e. the structural requirements for BAL binding are stricter than those for binding of the other ω-aminoaldehydes. This is because of the architecture and size of the active-site pocket where the bulky trimethylammonium group binds should allow for cation-π interactions with the four aromatic rings and, at the same time, should prevent steric clashes between the active site residues and the trimethylammonium group. Comparison of the aldehyde-binding sites of SoBADH and PsAMADH2 confirms this. Although an almost identical arrangement of the four aromatic residues can be observed in both, there is a subtle but critical difference between the spinach and pea enzyme responsible for the low affinity for BAL of the latter one: the size of the pocket formed by the residues equivalent to Tyr-160 and Trp-456 (SoBADH numbering) where the trimethylammonium group of BAL binds (Fig. 3). The residue behind the indole group of the tryptophan, an isoleucine in the pea enzyme and an alanine in SoBADH, determines the size of this pocket. The bulkier isoleucine pushes the tryptophan against the tyrosine, thus hindering the binding of BAL, as the kinetics of the SoBADH mutant A441I demonstrated. Our findings are consistent with the increase in BADH activity of the Y163A mutant of PsAMADH2 relative to the wild type enzyme (Kopěcný et al., 2011). Sequence alignments show that plant ALDH10 enzymes have only one of three different residues in the position of Ala-441, either an alanine, a cysteine or an isoleucine. On the basis of the structural and biochemical studies reported here, we propose that those ALDH10 enzymes that have an alanine or a cysteine at the position equivalent to Ala-441 of SoBADH are high-BAL-affinity
isoenzymes, which presumably are involved in synthesis of GB, while those ALDH10 enzymes that have an isoleucine in this position are low-BAL-affinity isoenzymes, which are likely involved in any of the other physiological functions of the plant ALDH10 enzymes (Fig. 1). The biochemical characterization of the ALDH10 isoenzymes from amaranth, spinach, barley, rice (Oryza sativa), pea, maize, and Arabidopsis thaliana supports our proposal that those of them having alanine or cysteine exhibit a high activity with BAL while those having isoleucine have a poor activity with this substrate (Supplemental Table S3). The exception is the isoleucine-containing isoenzyme from Avicennia, which was reported to have a high affinity for BAL and to be unable of using APAL and ABAL as substrates (Hibino et al., 2001). Interestingly, all known ALDH10s that we propose as high-BAL-affinity isoenzymes contain a tryptophan in the position equivalent to Trp-285 of SoBADH, while several low-BAL-affinity isoenzymes have an alanine, phenylalanine, proline or serine instead. This is consistent with our results of the kinetics of the W285A mutant.

Different ALDH10 genes have been reported to exist in the genome of several plants (McCue and Hanson, 1992; Ishitani et al., 1995; Wood et al., 1996; Legaria et al., 1998, Hibino et al., 2001; Bradbury et al., 2005) and the complete sequencing of several plant genomes has confirmed this trend. Some of them were considered alleles given their high similarity at the nucleotide and amino acid sequences (McCue and Hanson, 1992) but other may be true isoenzymes, not only because they might be located in different loci but mainly because they may perform different physiological functions, i.e. some may be involved in the synthesis of GB from BAL and others in oxidizing other ω-aminoaldehydes. To clarify this, we searched all available ALDH10 sequences of the NR collection of the NCBI protein database using SoBADH as a query. After eliminating the redundant sequences, we found that many species have two distinct ALDH10 sequences (Supplemental Table S3), which may correspond to two different isoenzymes. An examination of the ALDH10 sequences under the criterion of the residue occupying the position of Ala-441 in SoBADH indicated that some plants, such as amaranth (Amaranthus hypochondriacus), mangrove (Avicennia marina), barley (Hordeum vulgare), sorghum (Sorghum bicolor), Leymus chinensis, maize (Zea mays), and Zoysia tenuifolia have the two kinds of isoenzymes: one with alanine or cysteine in the position equivalent to Ala-441 of SoBADH and the other with isoleucine. Moreover, there are two ALDH10 genes that code for two
isoenzymes in the known plant genomes; although there are other sequences deposited in the NCBI protein database they are allelic variants or sequencing errors of these two. In some plants—such as maize, Zoysia, and sorghum—one of the two is the high-BAL-affinity isoenzyme and the other the low-BAL-affinity isoenzyme. In other plant species whose genomes are known—such as Arabidopsis thaliana, A. lyrata, Glycine max, Oryza sativa, Populus trichocarpa, and Solanum tuberosum—the two ALDH10 isoenzymes contain isoleucine and are therefore of the low-BAL-affinity kind. Phylogenetically, however, these two isoenzymes do not constitute two subfamilies within the ALDH10 family. It appears that they are the result of independent gene duplications that took place in different plant families.

Our phylogenetic analysis, described in the Experimental section, showed that plant ALDH10 sequences form a highly supported monophyletic clade, to the exclusion of bacterial ALDH10 and other plant ALDH sequences (bootstrap support for the monophyly of plant ALDH10: 100%; support for the monophyly of bacterial ALDH10: 99%; support for the monophyly of other plant ALDHs: 100%). Since these three groups of sequences formed well supported separate clades, either the bacterial ALDH10s or the plant ALDHs could be used to root the plant ALDH10 clade, thus revealing its branching order, which was the same regardless of whether the rooting was done with the bacterial ALDH10s or the plant ALDHs, and a sequence of mutations from the hypothetical ancestral sequence towards contemporary plant ALDH10s. The mapping of amino acid residues for the position corresponding to A441 in SoBADH indicates that isoleucine is the more common and ancient residue and that alanine tends to show up in gene duplicates in which the sister gene, if available, bears an isoleucine, whereas there are duplicates in which both copies bear an isoleucine. Furthermore, the bacterial ALDH10 sequences bear an isoleucine in the discussed position. Taken together, these observations strongly suggest that the ancestral plant ALDH10 gene coded for an isoleucine at the position homologous to A441 in SoBADH. A functional specialization seems to have occurred when in one of the two copies of the gene the isoleucine mutated into an alanine or a cysteine (or in the case of Vitis vinifera and Solanum tuberosum, into a valine).

It has been suggested that the main reason why some plants do no accumulate GB is the lack of a functional CMO or an inadequate supply of choline to the chloroplast (Nuccio et al., 1998). Our results indicate that the absence of the high-BAL-affinity ALDH10 isoenzyme may be a major limitation for GB biosynthesis in
plants. This is supported by our finding that the plant species in which one of the two isoenzymes is a high-BAL-affinity enzyme according to our criterion of possessing alanine or cysteine in the position of Ala441 of SoBADH, as well as other plants in which there have been found so far only one isoenzyme with alanine in this position, such as spinach and sugar beet (*Beta vulgaris*), or with cysteine, such as wheat (*Triticum aestivum*), have been reported as being GB-accumulators, whereas those plants that only have low-BAL-affinity isoenzymes are reported as lacking the ability to accumulate GB (Supplemental Table S3). Moreover, a functional CMO has only been found in species of Amaranthaceae that have the high-BAL-affinity isoenzyme: amaranth (Russell et al., 1998; Meng et al., 2001), orache (*Atriplex hortensis*) (Shen et al., 2002), spinach (Rathinasabapathi et al., 1997), sugar beet (Russell et al., 1998), whereas a non-functional gene was found in rice (Luo et al., 2007) and the recombinant CMO protein from *Arabidopsis* has no activity (Hibino et al., 2002). There are other CMO sequences deposited in GenBank, but it is not yet known whether the CMO proteins in these plants are functional or not.

Although there are no experimental data concerning the subcellular location of most of the ALDH10 enzymes, the presence of alanine or cysteine in position 441 of SoBADH correlates with a chloroplastic location in some of them while the presence of an isoleucine correlates with a peroxisomal location in others, but this is not a general rule (Supplemental Table S3). Thus, in Amaranthaceae the high BAL-affinity isoenzymes lack the carboxy-terminal tripeptide SKL that has been considered as a signal for transport into peroxisomes (Gould et al., 1988), and the chloroplastic location of the spinach enzyme has been experimentally determined (Weigel et al., 1986), consistent with CMO being also chloroplastic (Burnet et al., 1995). According to their C-terminus signal, the low-BAL-affinity isoenzymes, but not the high-BAL-affinity ones, from mangrove, barley, *L. chinensis* and wheat are predicted to be peroxisomal isoenzymes, but both kinds of isoenzymes from *Zoysia*, sorghum and maize have the peroxisomal signal. Interestingly a peroxisomal CMO has been recently reported in barley suggesting that the subcellular location of GB synthesis in this plant is not chloroplastic but probably cytosolic (Mitsuya et al., 2011). On the other hand, some low-BAL-affinity isoenzymes lack the peroxisomal signal. They may be located in leucoplasts, as it has recently been found for one of the two low-BAL-affinity isoenzymes from *Arabidopsis* (Missihoun et al., 2011). The rest of the low-BAL-affinity isoenzymes have an SKL or SKL-like C-terminus tripeptide. The
peroxisomal location has been proved for the low-BAL-affinity isoenzymes from barley (Nakamura et al., 1997) and from the second Arabidopsis isoenzyme (Missihoun et al., 2011), both of which have the C-terminus SKL tripeptide.

CONCLUSION

The first crystal structure of a plant BADH, that from spinach, together with site-directed mutagenesis studies provide for the first time the experimental evidence of the aromatic residues involved in binding of the trimethylammonium group of BAL in plant ALDH10 enzymes, and, importantly, of the main structural feature determining whether they accept betaine aldehyde as substrate: a non-active site amino acid residue located in the second sphere of interaction of the aldehyde bound inside the active site. If this is a small residue, alanine or cysteine, the enzyme will be a true BADH, whereas if this residue is an isoleucine, it pushes an active-site residue so that the cavity where the bulky trimethylammonium group of BAL binds is narrowed and the binding of BAL prevented. Consequently, the activity with BAL will be low and the enzyme can be described as an AMADH. This conclusion is confirmed by the previously reported biochemical characterization of some of these enzymes. A survey of the known plant ALDH10 sequences indicates that the presence or absence of the high-BAL-affinity ALDH10 isoenzyme in plants correlate with them being a GB-accumulator or non-GB-accumulator, respectively. Therefore, the lack of the high-BAL-affinity ALDH10 isoenzyme appears to be a major limitation for GB biosynthesis in plants.

EXPERIMENTAL

Chemicals and biochemicals

Betaine aldehyde chloride, the diethylacetals of APAL and ABAL, and NAD⁺ were obtained from Sigma-Aldrich Química, S.A. de C.V. (Toluca, México). The diethylacetal of TMABAL was synthesized following the method described (Vaz et al., 2000). APAL, ABAL and TMABAL were prepared freshly, hydrolyzing the corresponding diethylacetal forms following the method described by Flores and Filner (1985). The exact concentration of the resulting free aldehydes was determined.
in each experiment by determining the amount of NADH produced after their complete oxidation in the reaction catalyzed by SoBADH in the presence of an excess of NAD⁺.

**Construction of the expression plasmid**

The cDNA for SoBADH, a kind gift from Dr. Andrew D. Hanson (Horticultural Sciences Department, University of Florida, USA), was the template to obtain the His-tagged enzyme by PCR, using the forward primer 5´-AGCATATGGCGTTCCAATTCC-3´, which contains the starting codon and an NdeI restriction site, and the reverse primer 5´-CTCGAGAGGAGACTTGACC-3´, which corresponds to the 3´ end of the gene and contains a restriction site XhoI. The amplified DNA fragments were ligated into the pGEM-T easy vector (Promega) and selected in LB agar plates. Plasmids were purified and digested with NdeI and XhoI restriction enzymes and the fragment corresponding to the spinach *badh* gene purified and ligated into the corresponding sites of the pET28b⁺ vector (Invitrogen). The resulting plasmid, pET28-SoBADH, was used for the expression of full-length, N-terminal His-tagged, SoBADH.

For the expression of the recombinant proteins, cells of *E. coli* BL21(DE3) (Agilent) were grown at 37 ºC in 400 mL of Luria-Bertani broth, added with 50 mg mL⁻¹ of kanamycin, until the optical density at 600 nm reached 0.6. At this point, protein expression was induced by the addition of 0.1 mM isopropyl thio-β-D-galactoside (IPTG). The cells were allowed to grow for 4 h and harvested by centrifugation at 3800 g for 10 min. The pellet was suspended in 10 mL of 50 mM Hepes-KOH buffer, pH 7.5, containing 10 mM 2-mercaptoethanol and 10% (v:v) glycerol (Buffer A), and sonicated for 20 min. Cell debris was removed by centrifugation at 15000 rpm for 20 min and the supernatant was applied to a Q-Sepharose Fast Flow column (GE Healthcare) equilibrated with Buffer A. The column was washed with 10 volumes of the same buffer and the enzyme eluted with 300 mL of a linear KCl gradient from 0 to 350 mM in Buffer A. His-tagged enzymes were applied into a column packed with Protino Ni-TED resin (Macherey-Nagel, Düren, Germany) equilibrated with 50 mM Hepes-KOH buffer, pH 6.5, containing 10% (v:v) glycerol and 10 mM imidazole (Buffer B). The column was washed with the same buffer and the enzymes eluted by applying a linear gradient of imidazole.
from 10 to 250 mM in Buffer B. Imidazole excess was removed by centrifugal concentration, using Amicon Ultra 30 (Millipore), while Buffer B was replaced by Buffer A. To get rid of small contaminants, in the crystallization experiments a final step of purification through a Mono Q HR5 column (GE Healthcare) connected to a HPLC system (Waters) was included.

**Site-directed mutagenesis**

The plasmid pET28-SoBADH, containing the full sequence of the spinach *badh* gene and a N-terminal His-tag, was used as template for site-directed mutagenesis, which was performed via polymerase chain reaction (PCR) using the Quick Change XL-II Site Directed Mutagenesis system (Agilent) and the following mutagenic primers:

- **Y160A**, GATTAATATCCCATGGGAATGCCCACCCTCTAATGGCTAC (forward) and AGCCATTAGAAGTGCGCATTCATGCGGATATTGTTATCC (reverse);
- **W167A**, CCACCTTCAATGGCTACACCGGAAAATTGGCTCCAGCAGCTG (forward) and AAGTGCTGGAGCAATTTCGCTGTAGCCATTAGAAGTGGG (reverse);
- **W285A**, ACTATTTTGTGCTTTGCGACAAATGGTCAAAATATATGTAGG (forward) and ACATATTTGACCATTTTGCAGCCAAACAGCGAAAATATAGTCC (reverse);
- **W456A**, TTTGTTCAAGCTCTCGGCCAGGAGGCATCAAGCGTAGTG and CTACGCTTGGATGCTCCAGCAGGAGCTGAACAAAGCATG (reverse);
- **A441I**, GAAGGCTCTAGAAGTTGGAATTGTGTTTGTTGGTAAATTGTGC (forward) and TTGTGAACATCAACCACCCATGTTCTTCTTAGGCC (reverse);
- **A441C**, GAAGGCTCTAGAAGTTGGATGTGTTTGTTGGTAAATTGTGC (forward) and TTGTGAGCAATTACCCCAACACAATCTAATCTTAGAGCC (reverse).

The underlined portion of the oligonucleotides is the non-complementary mutagenic region. Mutagenesis was confirmed by DNA sequencing. The level of production of functional recombinant protein in *E. coli* was similar for the wild type SoBADH and its mutants.

**Activity assay and kinetic characterization of the wild-type and mutant SoBADH enzymes**

The specific dehydrogenase activities of wild-type SoBADH and its mutants were measured spectrophotometrically at 30 °C by monitoring the increase in the absorbance at 340 nm (ε = 6,220 M⁻¹ cm⁻¹) in a mixture (0.5 mL) consisting of 50 mM
HEPES-KOH buffer, pH 8.0, 1 mM EDTA, and 0.2 mM NAD$^+$ and variable concentrations of the aldehydes, or saturating concentrations of the aldehydes and variable NAD$^+$. The exact concentration of the aldehydes was determined by end-point assays using SoBADH and the standard assay conditions described below and the exact concentration of NAD$^+$ by its absorbance at 260 nm using a molar absorptivity of 18,000 M$^{-1}$ cm$^{-1}$ (Dawson et al., 1986). All assays were initiated by addition of the enzyme. Each saturation curve was determined at least in duplicate using enzymes from different purification batches. One unit of activity is defined as the amount of enzyme that catalyses the formation of 1 μmol of NADPH per min under our assay conditions.

Kinetic data were analyzed by non-linear regression calculations using a Michaelis-Menten equation that includes terms in the numerator and denominator to account for partial substrate inhibition (Equation 1):

$$v = \frac{V_{\text{max}}[S](1 + \beta[S]/K_{\text{IS}})}{K_m + [S](1 + [S]/K_{\text{IS}})}$$  \hspace{1cm} (1),

where $v$ is the experimentally determined initial velocity, $V_{\text{max}}$ is the maximal velocity, $[S]$ is the concentration of the variable substrate, $K_m$ is the concentration of substrate at half-maximal velocity, $K_{\text{IS}}$ is the substrate inhibition constant, and $\beta$ is the factor that describes the effect of substrate inhibition on $V_{\text{max}}$. For the estimation of $V_{\text{max}}/K_m$ values, and their associated standard errors (S.E.), a modified form of Equation 1 (Equation 2) was used:

$$v = \frac{V_{\text{max}}K_A[S](1 + \beta[S]/K_{\text{IS}})}{V_{\text{max}} + K_A[S](1 + [S]/K_{\text{IS}})}$$  \hspace{1cm} (2),

where $K_A$ is $V_{\text{max}}/K_m$. In those cases where no substrate inhibition was observed within the substrate concentration range used in the experiment the data were fitted to the Michaelis-Menten equation.

Two estimated values for a given kinetic parameter determined for the wild-type and a mutant enzyme, or for two different amino aldehyde substrates, were considered significantly different at 99% confidence levels when the estimated values had confidence intervals that did not overlap. Confidence intervals were computed multiplying the S.E. of the estimated value by a factor that depends on the confidence level chosen and on the number of degrees of freedom, which equals the number of data points minus the number of parameters that were fit.

Protein concentrations were determined spectrophotometrically, using the molar absorptivity at 280 nm deduced from the amino acid sequence by the method of
Gill and von Hippel (1989): 43,200 M⁻¹ cm⁻¹ for the wild-type, A441I and A441C enzymes; 40,450 M⁻¹ cm⁻¹ for the single tryptophan mutant enzymes (W167A, W285A and W456A); and of 42,455 M⁻¹ cm⁻¹ for the Y160A mutant enzyme.

**Crystallization, structure solution and refinement**

Crystals of wild-type SoBADH were obtained by the hanging-drop technique under anaerobic conditions. Prior to crystallization, wild-type SoBADH at a concentration of 20 mg/ml was incubated with 2 mM NAD⁺ for 15 min at room temperature. Subsequently, 2 µl of protein solution was mixed with 2 µl of reservoir solution, which contained 85 mM Tris-HCl, pH 8.5, 0.17 M sodium acetate trihydrate, 25.5 % (w:v) PEG 4000 and 15 % (v:v) glycerol (solution 22 of the crystal screen Cryo of Hampton Research, Aliso Viejo, CA, USA). Bar-shaped crystals appeared at 18 °C after 48 h and grew to their final dimensions within one week. The crystals were cooled under nitrogen stream at 100 K during data collection. Synchrotron data were collected at the National Synchrotron Light Source (Upton, NY, USA) on beam line X6a. The data were indexed with Mosflm (Leslie, 1992), integrated using XDS (Kabsch, 2010) and scaled and truncated with programs from the CCP4 suite (Collaborative, 1994).

Before phases were obtained, SoBADH diffraction data analysis suggests a triclinic symmetry, which was probed after phases were determined with molecular replacement. A clear solution for the molecular replacement was obtained with the program Phaser (McCoy et al., 2007) using the coordinates of the BADH from *Staphylococcus aureus* (SaBADH) (PDB code 3ED6) as a starting model. Two dimers were found in the asymmetric unit, however the low data / parameters ratio, because of the resolution of the diffraction, compromise a free refinement of all the components in the asymmetric unit. To avoid an over refinement, non-crystallographic symmetries (NCS) were applied, starting with constrained NCS, moving to tight restrained NCS, loose restrained NCS and finishing the refinement releasing the NCS. Once the NCS were retired, alternating cycles of automatic and manual refinement were carried out with the standard protocols of Phenix (Adams et al., 2002) monitoring the Rwork and Rfree split during the whole process. The program Coot (Emsley and Cowtan, 2004) was used to analyze the electron density. Water molecules were automatically localized using Phenix (Adams et al., 2002) and Coot. Structural alignments were performed with Coot and PyMOL (DeLano, 2002).
Docking and surface electrostatic potential calculations

Aldehyde molecules were rigidly docked into the active site of the SoBADH and PsAMADH2 three-dimensional structure, so that the carbonyl oxygen makes the known interactions inside the oxyanion hole, using the PyMOL building mode and then energy-minimized using the GROMOS 96 force field potential (van Gunsteren et al., 1996) of the Swiss PDB Viewer software (Guex and Peitsch, 1997). The convergence criterion was a value of 0.05 kJ/mol for the averaged derivative. The trimethylammonium group of BAL was non-rigidly docked in to the SoBADH after removing NAD⁺ and glycerol molecules from the crystallographic structure using the PatchDock server (Schneidman-Duhovny et al., 2005) and then further refined with FireDock (Mashiach et al., 2008). The solution with the highest geometric-shape-complementarity score was used.

Surface electrostatics calculations of SoBADH were carried out with the Adaptive Poisson-Boltzmann Solver (APBS) (Baker et al., 2001) using the PDB 2PQR web portal (http://kryptonite.ncbi.nlm/pdb2pqr/). The PQR (per-atom charge and radius) file was generated using the PARSE force field. The PROPKA program (Li et al., 2005) was used to assign the protonation state of SoBADH at pH 7.5. The rendered electrostatic potential was visualized using the plug-in APBS of the PyMOL software (DeLano, 2002).

Retrieval and phylogenetic analysis of ALDH10 orthologs

To obtain a phylogenetically wide sampling of ALDH10 orthologs from plants, we performed a blastp search on the NR collection of the NCBI protein database using the amino acid sequence of SoBADH as query. The maximum number of target sequences was set at 100 and, in order not to exclude any candidate plant ALDH10, the expected threshold was set at 10; the scoring matrix used was BLOSUM62, with gap opening and gap extension costs of 11 and 1 respectively. All sequences retrieved belong to either viridiplantae or eubacteria. In order to determine whether different hits coming from a given species correspond to different isoenzymes, we performed a series of blastp searches on the protein databases of the following completely sequenced and annotated species: Zea mays, Oryza sativa (japonica) and Solanum tuberosum, with the same parameters as previously indicated. Only hits with an E-value higher than 10e-3 were excluded. The resulting sequences were added to the
first set and aligned using MAFFT (Katoh et al., 2002). A maximum likelihood phylogeny was constructed using RAxML (Stamatakis, 2006) on the CIPRES web portal (Miller et al., 2010) under the JTT+G substitution model. The resulting tree was rooted at the bacterial sequences, showing a clade containing all the plant ALDH10 isoenzymes and a second clade including other plant ALDHs. This phylogeny was used as a guide and the following criteria were applied to exclude sequences that could represent allelic variants or sequencing errors: (1) all sequences with associated activity information were kept; (2) when chromosome mapping information was available, a representative of every locus was kept; (3) for sequences that were 99% identical or differed only by single position mutations, only one representative was kept.

Protein Data Bank accession number

The coordinates and the structure factors for the structure of SoBADH in complex with NAD$^+$ have been deposited in the Protein Data Bank (www.rcsb.org) with the accession code 4A0M.

SUPPLEMENTAL DATA

Supplemental Figure S1. Fold and secondary structure elements of the dimeric SoBADH.

Supplemental Figure S2. Topology diagram of the SoBADH subunit.

Supplemental Figure S3. Section of a SoBADH monomer showing the aldehyde tunnel and the position of Glu-103, Asp-107, and Asp-110.

Supplemental Table S1. Data collection and refinement statistics for the SoBADH crystal.

Supplemental Table S2. Kinetic parameters of wild-type and mutant SoBADH enzymes using NAD$^+$ as variable substrate.

Supplemental Table S3. Plant isoenzymes of the ALDH10 family.
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Figure Legends

**Figure 1.** Physiological role of the reactions catalyzed by plant ALDH10 enzymes with ω-aminoaldehydes and 3-dimethylsulfoniopropionaldehyde as substrates. BAL, betaine aldehyde; APAL, 3-aminopropionaldehyde; ABAL, 4-aminobutyraldehyde; TMABAL, 4-trimethylaminobutyraldehyde; DMSPAL, 3-dimethylsulfoniopropionaldehyde; GB, glycine betaine; GABA, 4-aminobutyrate; TMBA, 4-trimethylaminobutyrate; DMSP, 3-dimethylsulfoniopropionate.

**Figure 2.** Aromatic residues involved in binding of BAL to SoBADH. A, Stereoview of the $2F_o-F_c$ map contoured at 1 σ showing the aromatic residues in the aldehyde binding site. B and C, Stereoviews of energy-minimized models of a BA molecule docked into the active site showing the most favorable position of the trimethylammonium group when the carbonyl oxygen is kept inside the oxyanion hole and the carbonyl carbon is trigonal. In C, a section of the SoBADH active site shows the surface electrostatic potentials (-10 kT/e red, 10 kT/e blue) of solvent-accessible molecular surface of the aldehyde and nucleotide entrance tunnels. Aromatic side-chains are shown as sticks with carbon atoms in black, oxygen in red, nitrogen in blue, and sulfur in yellow. The methyl groups are shown in green sticks. Distances of the methyl groups to the aromatic residues are given in angstroms and depicted as red dashed lines. The hydrogen bonds between the carbonyl oxygen of the aldehyde and the oxyanion groups, i.e. the side-chain amide nitrogen of Asn-159 and the main chain amide nitrogen of C295, are depicted as black dashed lines. These panels were generated using PyMOL (DeLano, 2002). D, Saturation curves of wild-type SoBADH (■, black) and the mutants Y160A (□, red), W167A (●, blue), W285A (▲, orange), and W456A (○, violet) with BAL, APAL, ABAL, and TMABAL and substrates. Assays were carried out at pH 8.0 and fixed 0.2 mM NAD⁺. The points are the experimentally determined values, and the lines drawn through these points are those calculated from the best fit of the data by non-linear regression to Equation 1. Other experimental details are described in the Experimental section.

**Figure 3.** Structural determinants for BAL discrimination in ALDH10 enzymes. A, B, and C, Comparison of the aldehyde-binding sites of SoBADH and PsAMADH2. A
and B, Transversal section of SoBADH and PsAMADH2 monomers (PDB codes 4A0M and 3IWJ, respectively) showing the catalytic tunnel and the narrowing of the active size region where the trimethylammonium group of BAL binds due to the different position of W459 (PsAMADH2) compared with that of W456 (SoBADH), as signaled by the red arrow. Also showed is the catalytic cysteine, which is in the “attacking” (‘a’) conformation in SoBADH and in the “resting (‘r’) conformation in PsAMADH2. Amino acid side-chains are shown as sticks with carbon atoms in black (SoBADH) or grey (PsAMADH), oxygen in red, nitrogen in blue, and sulfur in yellow. C, Molecular surface of the aromatic residues of SoBADH (beige molecular surface, black carbon atoms) and PsAMADH (grey molecular surface, pink carbon atoms) showing the steric clash (marked by a red arrow) in the latter enzyme with the trimethylammonium group, shown as green balls, of a modeled BAL molecule. In PsAMADH, Ile-444 pushes Trp-459 toward Tyr-163 while in SoBADH Ala-441 allows Trp-456 to be more distant from Tyr-160, thus leaving enough room between these two residues for a trimethylammonium group to bind. Images were generated using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, CA (Pettersen et al., 2004). D, Effects of mutation of residue Ala-441 to isoleucine or cysteine on the kinetic parameters of SoBADH with BAL as variable substrate. Enzyme assays were carried out as described in the Experimental section, and the data analyzed as described in Fig. 2D. E, Saturation curves of wild-type SoBADH (■, black) and the mutants A441I (◆, red) and A441C (◇, green) with BAL, APAL, ABAL, and TMABAL as substrates. Enzyme assays were carried out and the data analyzed as described in Fig. 1D.
The estimated parameter is significantly different to that determined with BAL at 99% confidence level. The estimated parameter is significantly different to that determined for the WT enzyme at 99% confidence level.

Table I. Kinetic parameters of wild-type and mutant SoBADH enzymes using different ω-aminoaldehydes as substrates

Initial velocities were obtained at 30 °C, pH 8.0 and 0.2 mM of NAD⁺. The kinetic parameters ± the standard errors were estimated by non-linear regression of the experimental data to equation 1 or 2, as appropriate. S.E. values were around 10% of the estimated kinetic parameters. $V_{\text{max}}$ and $K_m$ values are given as U/mg protein and μM, respectively. BAL, betaine aldehyde; APAL, 3-aminopropionaldehyde; ABAL, 4-aminobutyraldehyde; TMABAL, 4-trimethylaminobutyraldehyde; DMSPAL, 3-dimethysulfoniopropionaldehyde.

| Enzyme | BAL | APAL | ABAL | TMABAL |
|--------|-----|------|------|--------|
|        | $V_{\text{max}}$ | $K_m$ | $V_{\text{max}}/K_m$ | $V_{\text{max}}$ | $K_m$ | $V_{\text{max}}/K_m$ | $V_{\text{max}}$ | $K_m$ | $V_{\text{max}}/K_m$ |
| WT     | 5.4±0.1 b | 69±5 | 7.8±0.4 (×10⁻⁵) | 0.70±0.04 a | 2.6±0.3 a | 2.7±0.4 a (×10⁻¹) | 0.58±0.03 a | 5.5±0.6 a | 1.0±0.2 (×10⁻¹) | 1.2±0.0 a | 3.6±0.2 a | 3.4±0.1 a |
| Y160A  | 1.4±0.1 b | 9.7±1.2 (×10³) | 1.4±0.8 (×10⁻⁶) | 0.86±0.21 | 2.0±0.2 (×10⁻¹) | 4.4±0.4 (×10⁻³) | 0.34±0.02 b | 1.2±0.1 (×10⁻³) | 2.8±0.2 (×10⁻¹) | 0.68±0.02 b | 86±8 b | 7.9±0.6 |
| W167A  | 0.62±0.01 b | 2.9±0.2 (×10⁰) | 2.1±0.1 (×10⁻⁶) | 0.49±0.01 b | 3.6±0.3 (×10⁻³) | 1.4±0.1 b | 1.4±0.1 b | 31±6 b | 4.5±0.4 (×10⁻²) | 0.85±0.03 | 3.7±0.6 | 2.3±0.3 |
| W285A  | 3.4±0.4 b | 1.2±0.3 (×10⁰) | 2.8±0.3 (×10⁻⁵) | 0.14±0.01 b | 3.3±0.8 (×10⁻²) | 4.2±0.4 (×10⁻²) | 0.30±0.00 b | 2.3±0.2 b | 1.3±0.1 (×10⁻¹) | 0.47±0.01 b | 1.8±0.2 | 2.6±0.3 |
| W456A  | 1.7±0.2 b | 2.4±0.5 (×10⁰) | 7.0±0.7 (×10⁻⁵) | 0.52±0.08 | 3.9±0.8 (×10⁻²) | 1.3±0.0 (×10⁻¹) | 0.19±0.03 b | 4.3±0.6 | 4.4±0.0 (×10⁻³) | 0.19±0.02 b | 1.5±0.4 b | 1.3±0.2 b |

*The estimated parameter is significantly different to that determined with BAL at 99% confidence level. b The estimated parameter is significantly different to that determined for the WT enzyme at 99% confidence level.
The estimated parameter is significantly different to that determined for the WT enzyme at 99% confidence level.

Table II. Kinetic parameters of wild-type and mutant SoBADH enzymes using different ω-aminoaldehydes as substrates

Initial velocities were obtained at 30 °C, pH 8.0 and 0.2 mM of NAD⁺. The kinetic parameters ± the standard errors were estimated by non-linear regression of the experimental data to Equation 1 or 2, as appropriate. $V_{\text{max}}$ and $K_m$ values are given as U/mg protein and μM, respectively. BAL, betaine aldehyde; APAL, 3-aminopropionaldehyde; ABAL, 4-aminobutyraldehyde; TMABAL, 4-trimethylaminobutyraldehyde; DMSPAL, 3-dimethylsulfoniopropionaldehyde.

| Enzyme | BAL $V_{\text{max}}$ | BAL $K_m$ | BAL $V_{\text{max}}/K_m$ ($\times10^{-3}$) | APAL $V_{\text{max}}$ | APAL $K_m$ | APAL $V_{\text{max}}/K_m$ ($\times10^{-3}$) | ABAL $V_{\text{max}}$ | ABAL $K_m$ | ABAL $V_{\text{max}}/K_m$ ($\times10^{-3}$) | TMABAL $V_{\text{max}}$ | TMABAL $K_m$ | TMABAL $V_{\text{max}}/K_m$ ($\times10^{-3}$) |
|--------|------------------|----------|---------------------------------|------------------|----------|---------------------------------|------------------|----------|---------------------------------|------------------|----------|---------------------------------|
| WT     | 5.4±0.1          | 69±5     | 7.8±0.4 ($\times10^{-3}$)       | 0.70±0.04        | 2.6±0.3  | 2.7±0.4 ($\times10^{-3}$)       | 0.58±0.03        | 5.5±0.6  | 1.0±0.2 ($\times10^{-3}$)       | 1.2±0.0          | 3.6±0.2  | 3.4±0.1 ($\times10^{-3}$)       |
| A441I  | 0.77±0.02        | 1.6±0.2  | 4.7±0.4 ($\times10^{-3}$)       | 0.65±0.01        | 2.5±0.1  | 2.6±0.8 ($\times10^{-3}$)       | 1.05±0.06        | 3.8±0.5  | 2.7±0.2 ($\times10^{-3}$)       | 0.7±0.21         | 2.8±0.3  | 2.5±0.2 ($\times10^{-3}$)       |
| A441C  | 5.1±0.6          | 120±20   | 4.3±0.7 ($\times10^{-3}$)       | 0.78±0.16        | 2.8±0.6  | 2.7±0.2 ($\times10^{-3}$)       | 0.58±0.05        | 1.8±0.2  | 3.2±0.5 ($\times10^{-3}$)       | 0.88±0.22        | 2.7±0.2  | 3.2±0.2 ($\times10^{-3}$)       |

*The estimated parameter is significantly different to that determined for the WT enzyme at 99% confidence level.
