Site-Directed Mutagenesis of a Serine Residue in Cinnamyl Alcohol Dehydrogenase, a Plant NADPH-Dependent Dehydrogenase, Affects the Specificity for the Coenzyme†

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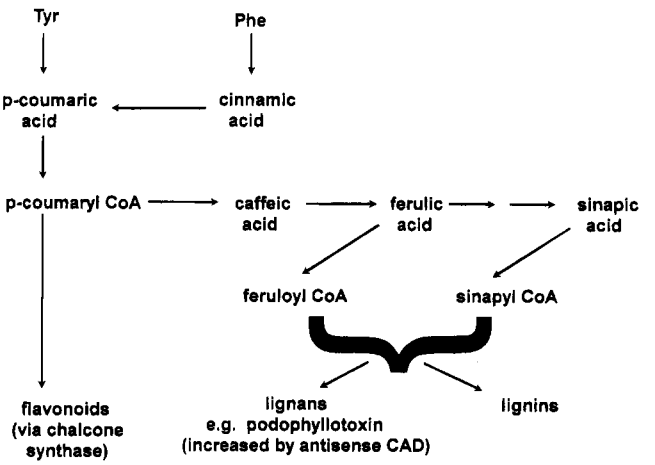
ABSTRACT: Using recombinant cinnamyl alcohol dehydrogenase isoform 2 (CAD2, EC 1.1.1.195), an NADPH-dependent aromatic alcohol dehydrogenase involved in lignification in vascular plants, we have investigated the detailed steady-state kinetic mechanism of CAD2 and the role of a serine residue in determining the cofactor specificity of CAD2. Site-directed mutagenesis (S212D) and overexpression of the WT and mutant S212D forms of CAD2 in *Escherichia coli*, followed by kinetic studies on the purified WT and mutant proteins, confirmed the involvement of S212 in recognizing the phosphate group of NADPH and provided information on the structural requirements for NADPH specificity. From substrate kinetic patterns and product inhibition studies both WT and S212D mutant forms of CAD2 have been shown to follow rapid equilibrium random bireactant kinetics with the value of the interaction factor (α) for WT (0.25) being significantly less than that for S212D CAD2 (0.45). The changes in binding energy arising from the mutation on the binding of the 2′-phosphate site of the coenzyme were assessed. A marked degree of physical interaction was detected between the enzymatic binding sites of the coniferyl alcohol substrate and the 2′-phosphate binding region, which are quite distant in the three-dimensional structure. The inhibition by 2′,5′-ADP and 5′-AMP was found to be weak for both WT and S212D CAD2. Strong substrate inhibition was detected for CAD2, and its implications for plant physiological studies were assessed. The overall catalytic efficiency [$k_{cat}/(K_m^A \times K_m^B)$] for CAD2 with $NADP^+$ as coenzyme is decreased 2.2×10^3-fold by the single mutation S212D, while there is no significant change in this parameter with NAD^+ as coenzyme. The S212D mutation allows CAD2 (normally using NADPH) to function with NADH as coenzyme at 1/25 the rate of WT with NADPH, indicating that CAD2 would provide a good basis for any multiple-mutation engineered switch in coenzyme usage from NADPH to NADH to produce a metabolic probe of plant physiology.

Lignins, the second most abundant biopolymers after cellulose, account for up to 30% of the terrestrial biomass and play an important role in mechanical support, water retention, and defense mechanisms in higher plants. Lignins are formed by the polymerization by cell wall peroxidases or laccases of three phenolic monomeric units, coniferyl, sinapyl, and *p*-coumaryl alcohols. These monolignols are derived from general phenylpropanoid metabolism (Scheme 1) followed by two steps (eq 1) specific to lignin biosynthesis, *viz.*, the reduction of esters of cinnamoyl coenzyme A to cinnamaldehydes by cinnamoyl-CoA reductase (CCR,[1] EC 1.2.1.44) followed by their reduction to the corresponding alcohols by cinnamyl alcohol dehydrogenase (CAD, EC

Scheme 1. Common Phenylpropanoid Pathway



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[1] Abbreviations: ADH, alcohol dehydrogenase; CAD, cinnamyl alcohol dehydrogenase; CCR, cinnamyl-coenzyme A reductase; IPTG, isopropyl β-thiogalactopyranoside; HLADH, horse liver alcohol dehydrogenase; PMSF, phenylmethanesulfonyl fluoride; WT, wild type.

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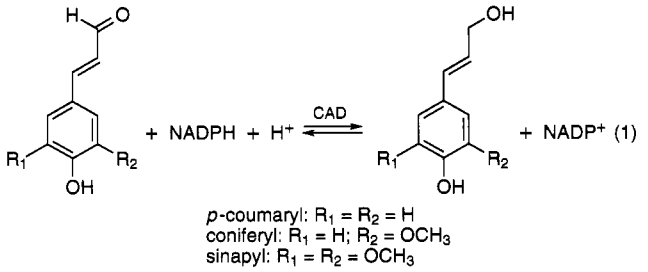


1.1.1.195). There is considerable current interest in altering plant lignification (both qualitatively and quantitatively) by manipulation of CAD activity either by means of chemical inhibition (Grand et al., 1985; Baltas et al., 1988) or more recently by antisense strategies (Halpin et al., 1994).

CAD is a polymorphic enzyme, at least two isoforms having been identified for *Eucalyptus* (Goffner et al., 1992). Sequence analysis of the gene coding for the CAD2 isoform from *Eucalyptus gunnii* Hook (Grima-Pettenati et al., 1993) showed the CAD derived from it to be sufficiently homologous to a range of dehydrogenases to allow calculation of a three-dimensional structure of CAD2 by homology modeling using the crystallographic coordinates of HLADH (McKie et al., 1993). CAD is an unusual alcohol dehydrogenase on the basis of its specificity for aromatic alcohols/aldehydes (eq 1), but the molecular origins of its specificity have not yet been fully explored, although initial molecular modeling has provided some insights (McKie et al., 1993). In contrast to most other alcohol dehydrogenases, which use NADH, CAD is NADPH-dependent. Sequence alignments show that the NADPH-dependent CAD2 from *Eucalyptus* (Grima-Pettenati et al., 1993), as well as that from other species [*viz.*, spruce (Galliano et al., 1993), loblolly pine (O'Malley et al., 1992), tobacco (Knight et al., 1992), poplar, and alfalfa (Van Doorsselaere et al., 1995) (and references therein)], possesses a serine residue (S212 in *Eucalyptus*) at the site corresponding to an aspartate residue (D223) in the NADH-dependent HLADH. This latter residue has been shown by crystallographic data to be involved in hydrogen bonding to the ribosyl 2′-OH group of the NADH cofactor (Eklünd et al., 1984). This residue or its equivalent has been studied previously for several dehydrogenases (all of which were NAD-dependent) as well as glutathione reductase (which is NADP-dependent) (Thomas et al., 1994; Novotny et al., 1984; Feeney et al., 1990; Mittl et al., 1993; Ganzhorn et al., 1987; Perham, 1991; Fan et al., 1991; Chen et al., 1991; Miyazaki & Oshima, 1994; Huang et al., 1990; Clermont et al., 1993; Nishiyama et al., 1993; Bocanegra et al., 1993; Untucht-Grau et al., 1981). In the present study we have used site-directed mutagenesis for CAD2, an NADP-dependent alcohol dehydrogenase, to elucidate the role of residue S212 in the binding of $NADP^+$, by replacing it with an aspartic acid residue (Figure 1), which should select against NADPH binding primarily because of electrostatic repulsion, on the basis of analysis of the homology model of *Eucalyptus* CAD (McKie et al., 1993). As a first step, we had to develop rapid, efficient purification protocols for both WT and mutated recombinant CAD2s. This then enabled a detailed comparison of their kinetic characteristics. We also describe the presence of substrate inhibition for CAD2, indicating a modification of the assay to allow for the possibility of isoform- or species-dependent substrate inhibition of CAD.

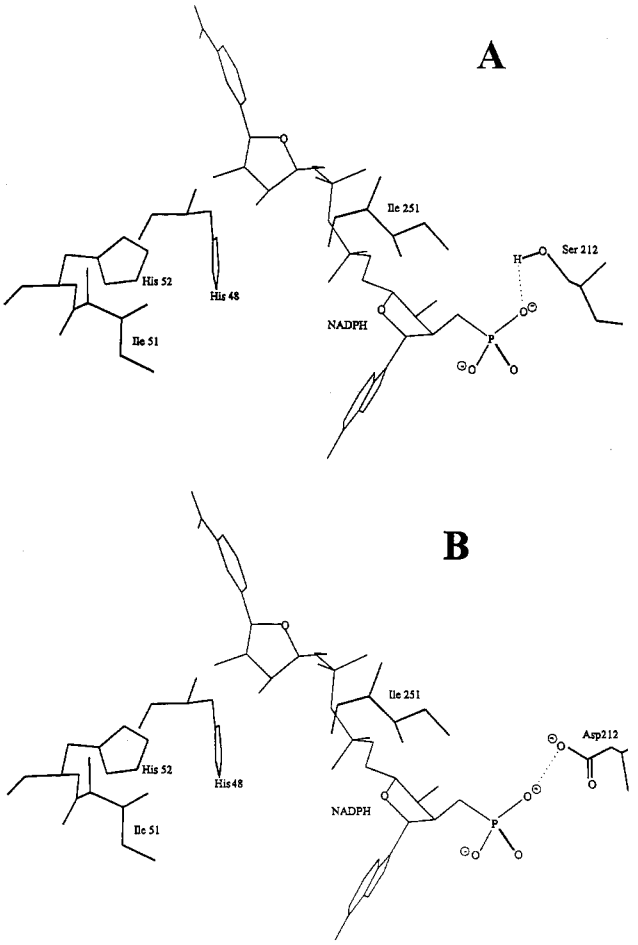


FIGURE 1: Molecular graphics picture of S212D mutation site of *Eucalyptus* CAD with protein side chains shown in boldface lines and cofactors shown in normal lines. View A shows the relative location of the side chain of serine 212 with the hydrogen atom of the OH group 0.231 nm from the O^- of the 2′-phosphate group of NADPH. View B shows the very close approach that would occur for the S212D mutant of CAD (with the CO_2^- of Asp212 lying almost on top of the PO_3^{2-} group of NADPH, we have moved Asp212 to allow visualization) and the high degree of both steric and electrostatic conflict that would result. These conflicts do not occur when NADPH is replaced in the molecular graphics model by NADH (not shown).

MATERIALS AND METHODS

Materials. Coniferyl alcohol, $NADP^+$, NADPH, NAD^+, NADH, 5′-AMP, and 2′5′-ADP were from Sigma Chemicals. Coniferaldehyde was from Aldrich. All primers and restriction enzymes were from Biolabs or Eurogentec. The Taq polymerase and lysosyme used were from Bioprobe. IPTG and Benzonase (nonspecific nuclease) were from Eurogentec. Q-Sepharose FastFlow and MonoQ columns were from Pharmacia.

Site-Directed Mutagenesis and Construction of a cDNA Coding for the Mutant S212D CAD2. The construction of the expression vector producing wild-type recombinant CAD2 was previously described (Grima-Pettenati et al., 1993). The S212D mutation was introduced into the CAD2 cDNA by PCR using the method described (Bowman et al., 1990). An oligonucleotide, 5′-GTGATAAGCGATTCT-GATAGAAG-3′, was used to change the codon TCT encoding serine 212 into codon GAT coding for aspartic acid (underlined). The other oligonucleotide used was the M13 reverse primer (Biolabs) located on the pGEM vector

downstream from the stop codon of the CAD2 cDNA. PCR was performed using 5 ng of plasmid template [pEUCAD2 (Grima-Pettenati et al., 1993)] and 20 pmol of each primer. The samples were incubated in a DNA thermal cycler (Perkin-Elmer/Cetus) using the following conditions: denaturation at 94 °C for 1 min, annealing at 60 °C, and extension at 72 °C for 2 min 30 s with Taq polymerase (Bioprobe). The last cycle had a 10-min extension step. The PCR products were purified onto glass beads (Geneclean, Bio 101). A second PCR was performed using as a template an aliquot of the first PCR reaction mixture in order to amplify the minor product, i.e., the complete mutated cDNA. This latter PCR reaction used the M13 reverse primer (15 pmol) and the oligonucleotide S1 (25 pmol), which introduced an *Nde*I site at the ATG start codon of the CAD2 cDNA (Grima-Pettenati et al., 1993). The PCR product was then gel-purified, digested by *Nde*I and *Eco*RI, and subsequently ligated into the *Nde*I−*Eco*RI-linearized pT7-7 vector. The resulting construct, containing the S212D mutated cDNA, was transferred to the bacterial strain *Escherichia coli* BL21 (DE3). The complete cDNA was sequenced to verify the incorporation of the S212D mutation and the absence of other undesired mutations, using the dideoxy chain termination method (Sanger et al., 1977).

High-Level Expression of the CAD2 cDNAs in E. coli. Large-scale production of recombinant CAD2, both wild type and mutant, was started by inoculating 2.4 L of Luria broth medium, containing 1 mM zinc chloride and 50 μg/mL ampicillin, with 24 mL of an overnight culture of BL21 cells transformed with the pT7-7 vector harboring the appropriate CAD2 cDNA. The cells were cultured at 37 °C until an absorbance of 0.8−1.0 at 600 nm was reached, at which point the IPTG inducer was added to a final concentration of 1 mM. The incubation temperature was reduced to 15 °C to limit the formation of inclusion bodies, and the bacteria were grown for a further 16 h and then harvested by centrifugation.

Preparation of Soluble Protein Extracts from E. coli. Pelleted cells were resuspended in cold 20 mM Tris/HCl buffer, pH 7.5, containing 10% glycerol, 5 mM DTT, 0.1% Nonidet P40, 1 mM PMSF, 1 mM EDTA, and 5 μg/mL leupeptin. Lysozyme was added to 2 mg/mL, and the cells were incubated at 4 °C until lysis had occurred. Nuclease (Benzonase, Eurogentec) was added to a concentration of 50 units/mL, and the mixture was incubated at 4 °C until the cell lysate became less viscous (usually 15 min). Cell debris was pelleted by centrifugation.

Purification of Wild-Type and Mutant CADs. The cleared cell lysate after centrifugation was applied to a column of Q-Sepharose FastFlow (100 × 2.6 cm), preequilibrated with buffer A (pH 7.5, 20 mM Tris/HCl, 5mM DTT, and 2% ethylene glycol) using a flow rate of 10 mL/min. After the sample was applied, the column was washed with 10 column volumes of buffer A, followed by 10 column volumes of buffer A containing 100 mM NaCl. CAD2 was eluted with 200 mM NaCl in buffer A. Recombinant WT and mutant CAD2s were further purified by pooling samples containing activity and precipitating with 80% ammonium sulfate. The precipitate was centrifuged (27000*g*, Beckman JA20), and the pellet was resuspended in 100 mM Tris/HCl buffer, pH 7.5, containing 5 mM DTT and 5% ethylene glycol. The WT and mutant CAD2s, after desalting using a PD-10 column (Pharmacia), were applied to a MonoQ column (HR 5/5). The sample was diluted in buffer A (1/5, v/v) and applied to the MonoQ column, preequilibrated with the same buffer, at a flow rate of 1 mL/min. After the column was washed with 10 column volumes of the same buffer, the CAD2 was eluted with a gradient of 20−200 mM NaCl (total volume, 50 mL).

After denaturing polyacrylamide gel electrophoresis (Laemmli, 1970), proteins were stained with silver nitrate (Damerval et al., 1987). Total protein content of samples was determined using the Bio-Rad assay based on the method of Bradford (Bradford, 1976).

Determination of Kinetic Parameters of the Recombinant Wild-Type and Mutant CAD. Recombinant wild-type and S212D CAD2 were assayed spectrophotometrically by both the oxidation of coniferyl alcohol and the reduction of the corresponding aldehyde. The reverse reaction (alcohol to aldehyde) was monitored spectrophotometrically by the increase in absorbance at 400 nm due to the production of the coniferaldehyde. The forward reduction was determined by the change in absorbance at 340 nm due to the disappearance of coniferaldehyde and NADPH as described by Wyrambik and Grisebach (1975). The assay was carried out at 30 °C in 0.5 mL of reaction mixture containing, for the reverse reaction, 100 mM Tris/HCl, pH 8.8, 100 μM coniferyl alcohol, 200 μM $NADP^+$ or NAD^+, and 5−20 μL of protein extract or, for the forward reaction, 100 mM KH_2PO_4/Na_2HPO_4, pH 6.25, 50 μM coniferaldehyde, 200 μM NADPH or NADH, and 5−20 μL of protein extract.

The following molar extinction coefficients were used: ϵ_{400} coniferaldehyde = 18.5×10^3 M^{-1} cm^{-1} at pH 8.8; ϵ_{340} coniferaldehyde = 15.8×10^3 M^{-1} cm^{-1}; ϵ_{340} NADPH = 6.2×10^3 M^{-1} cm^{-1}; ϵ_{340} NADH = 6.22×10^3 M^{-1} cm^{-1} at pH 6.25. To determine whether there were any competitive dehydrogenase activities in the final protein extract, controls were performed under the same conditions as the assay reaction omitting in turn substrate or cofactor from the assay; no activity was detected.

Inhibition of the reaction by adenosine 5′-monophosphate and adenosine 2′5′-diphosphate was studied kinetically using the conditions described above. Values of K_i and I_{50} (the concentration of inhibitor giving 50% inhibition) were measured for both directions of the enzymatic reaction, i.e., coniferyl alcohol oxidation and coniferaldehyde reduction. From the I_{50} values determined, suitable ranges of inhibitor concentration were chosen for detailed K_i and inhibition studies. All kinetic measurements were performed at least three times, and mean values were used for subsequent calculation.

Data were analyzed by direct nonlinear regression analysis using the Enzfitter program, written by R. J. Leatherbarrow and distributed by Elsevier Biosoft. An initial analysis of three diagnostic plots of inhibition type was made: $1/V_o$ *versus* $1/[S_o]$ at varying $[I_o]$, $1/V_o$ *versus* $[I_o]$ at various $[S_o]$, and $[S_o]/V$ *versus* $[I_o]$ at various $[S_o]$. From these, further diagnostic plots were chosen to allow a choice of kinetic model. Values of K_i were determined by assuming the appropriate kinetic model for inhibition. Further kinetic studies were then performed to confirm the kinetic model (Segel, 1975).

Molecular Model of CAD2. The model used was that previously described (McKie et al., 1993) and was based on the X-ray coordinates of HLADH with input also from other dehydrogenases, all of which showed sequence similarity in the 24−27% range. As described (McKie et al., 1993), the

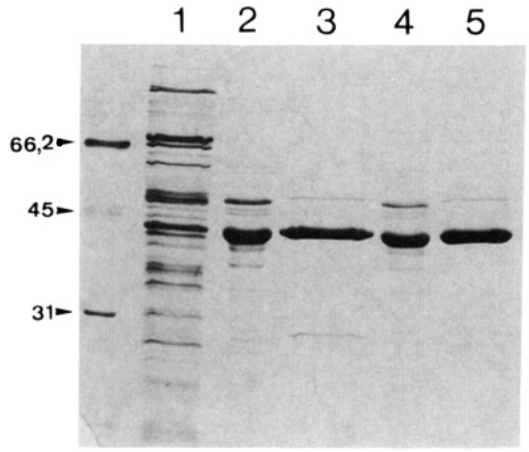


FIGURE 2: SDS−gel electrophoresis of the two purification steps for recombinant WT and S212D CAD: crude extract (lane 1); Q-Sepharose chromatography of WT CAD (lane 2); MonoQ chromatography of WT CAD (lane 3); Q-Sepharose chromatography of S212D mutant enzyme (lane 4); MonoQ chromatography of S212D CAD (lane 5). Proteins were visualized by staining with silver nitrate.

derived homology model gave satisfactory explanations of the molecular basis of substrate binding, the coenzyme specificity, and the stereospecificity of hydride transfer. The Rossmann fold was found to be comparable to that in other dehydrogenases, but with a kinked α-helix (corresponding to helix α_4 of HLADH) (McKie et al., 1993). The model also successfully predicted novel classes of inhibitor for CAD (McKie et al., 1993). Thus confidence in the model is established.

RESULTS

Purification of WT and Mutated (S212D) CAD2 Enzymes. Recombinant CAD2 was purified with a first step which exploited its negative charge at pH 7.5. Ion-exchange chromatography on Q-Sepharose FastFlow led to a 5.5−6-fold purification of both wild-type and S212D mutant CAD2. In other studies which are not described here, wild-type CAD2 was then purified to homogeneity by affinity chromatography on 2′5′-ADP Sepharose which uses its ability to bind $NADP^+$ (K. Kennedy, unpublished results). However, attempts to purify the mutant CAD2 on the same affinity column were encouragingly unsuccessful with no binding to the matrix occurring, the activity being recovered in the column wash before the eluting gradient was applied. Apparently, the S212D mutation had disrupted interactions with the 2′5′-ADP ligand. A second ion-exchange step was therefore adopted for the mutant CAD2 using a MonoQ column, which led to a 2-fold purification, with 12.3 and 13 as purification factors for WT and mutant proteins, respectively. The same protocol of purification was used for both WT and S212D CAD2 to allow kinetic studies on enzyme with the same history and degree of purification, in order that the specific activities and results obtained could be directly compared. After this second MonoQ step, the WT and mutant S212D CAD2 were shown to be greater than 90% homogeneous as determined by SDS−PAGE (see Figure 2).

Physicochemical and Kinetic Characteristics of WT and S212D CAD2. The specific activities of recombinant wild-type and S212D CAD2, after the last step of the purification, are given in Table 1 for both $NADP^+$ and NAD^+ as cofactors. The specific activity of recombinant CAD2 (78.4 nkat/mg) was of the same order as that reported (91.7 nkat/mg) for native CAD2 (Goffner et al., 1992). The specific activity of the WT enzyme was 14.2-fold greater with $NADP^+$ as cofactor than with NAD^+. The most striking effect of the S212D mutation was observed at the specific activity level (Table 1). The specific activity of S212D with $NADP^+$ as cofactor dropped by a factor of 2.8 as compared to the WT, whereas the specific activity with NAD^+ remained roughly the same as the WT, resulting in a much less pronounced preference for $NADP^+$ ($NADP^+/NAD^+$ = 4.3). The values of the kinetic parameters (k_{cat}, K_m, and k_{cat}/K_m) are recorded in Table 2 for WT and S212D CAD2 for both directions of the reaction. For $NADP^+$ the value of K_m was increased 90-fold for the mutated enzyme, while there was no significant difference for NAD^+ between WT and S212D enzymes. There was a parallel, small increase in the value of K_m of coniferyl alcohol using $NADP^+$ as coenzyme. In the case of NADPH, the K_m value for the mutated enzyme was about 27-fold higher than the K_m for WT CAD2. Detailed kinetic studies with NADH were not feasible due to the high K_m value for this coenzyme. The turnover numbers (k_{cat}) were not affected by the mutation, while the k_{cat}/K_m ratio for the mutated enzyme with NADP(H) as cofactor was decreased 25-fold in the forward direction (coniferaldehyde reduction) and 130-fold in the reverse direction.

Table 1: Specific Activities at 30 °C for Recombinant WT Cinnamyl Alcohol Dehydrogenase (WT) and the S212D Mutant Enzyme[a]

	specific activity (nkat/mg)[b]		
enzyme	$NADP^+$	NAD^+	$\frac{NADP^+}{NAD^+}$
WT	78.4 ± 2.6	5.5 ± 0.9	14.2
S212D	27.8 ± 4.1	6.5 ± 1.1	4.3

[a] The medium was pH 8.8, with the concentration of coniferyl alcohol at 0.1 mM and the nicotinamide cofactor concentration at 0.2 mM. [b] 1 kat is the amount of enzyme activity that converts 1 mol of substrate per second.

To determine whether the kinetic mechanism of the enzyme was modified by the mutation, diagnostic plots were carried out, *viz.*, the double-reciprocal plot of initial velocities with the $NADP^+$ concentration changing (fixed substrate, coniferyl alcohol) and Eadie−Scatchard, Hanes−Woolf and Woolf−Augustinsson−Hofstee plots. These are shown for WT CAD2 and S212D mutant CAD2 in Figure 3, panels A−D, respectively. The reverse double-reciprocal plot of initial velocities with coniferyl alcohol as changing substrate concentration and fixed $NADP^+$ concentration was also measured and was symmetric with Figure 3A for WT CAD2.

Plots of product inhibition for WT CAD2 and S212D CAD2 are shown in Figure 4, which shows the effects of coniferaldehyde concentration on coniferyl alcohol oxidation.

Studies were also carried out of the inhibition by adenosine 2′5′-diphosphate (2′5′-ADP) and adenosine 5′-monophosphate (5′-AMP), which are $NADP^+$ and NAD^+ analogues, respectively; see Table 3. Weak inhibition was detected for 5′-AMP with the I_{50} value for WT CAD2 being 1.58 mM with $NADP^+$ as coenzyme. When NAD^+ was used as cofactor, 5′-AMP was found to inhibit both WT and S212D CAD2 2.5-fold more strongly than with $NADP^+$. Using all

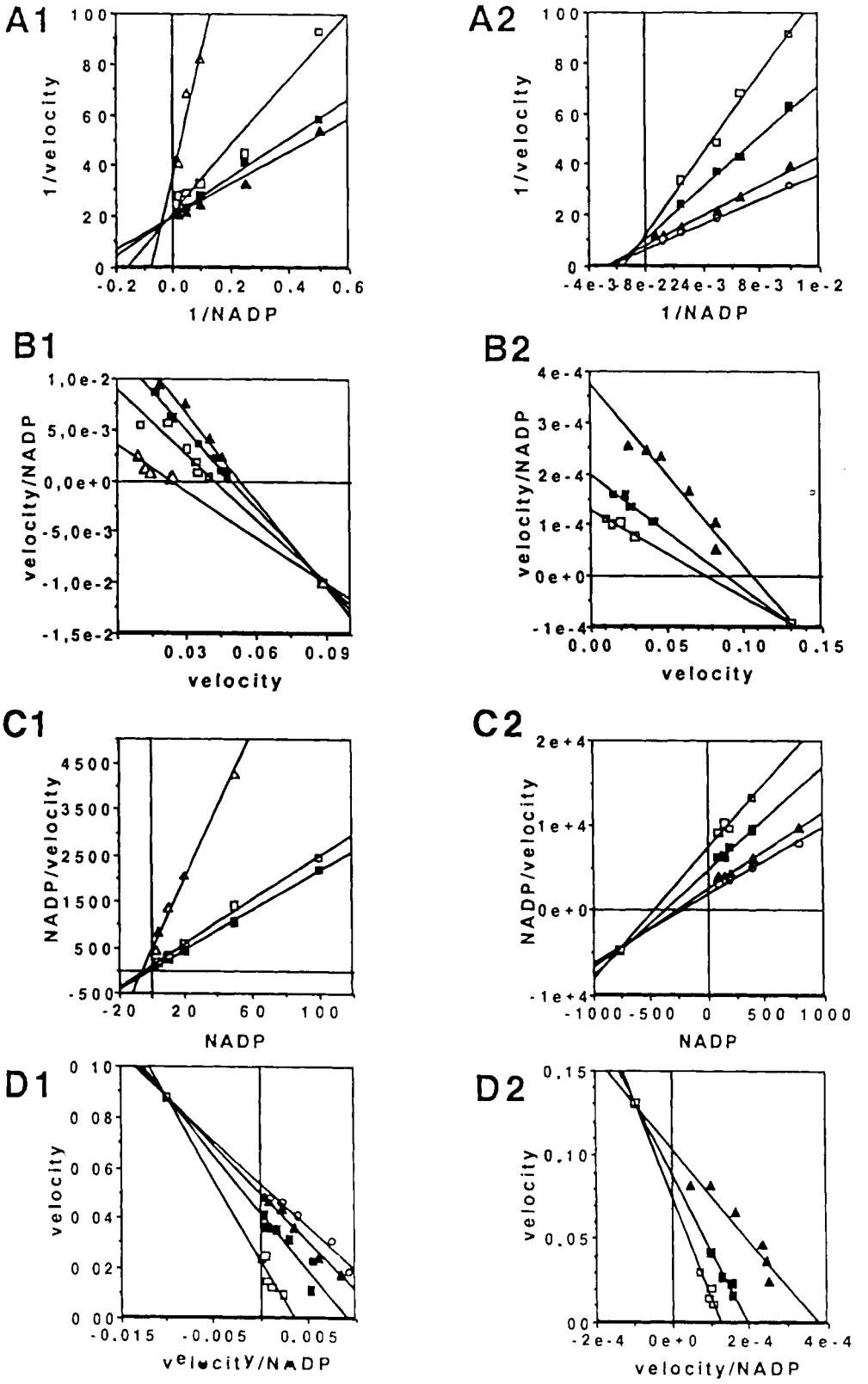


FIGURE 3: Diagnostic plots for recombinant WT cinnamyl alcohol dehydrogenase (A1−D1) and the S212D enzyme (A2−D2). (A) is the double-reciprocal plot of 1/velocity *versus* 1/[$NADP^+$] (fixed substrate, coniferyl alcohol). (B) is the Eadie−Scatchard plot of velocity/[$NADP^+$] *versus* velocity. (C) is the Hanes−Woolf plot of [$NADP^+$]/velocity *versus* velocity. (D) is the Woolf−Augustinsson−Hofstee plot of velocity *versus* velocity/[$NADP^+$]. Each line represents the coniferyl alcohol concentration indicated as follows: △, 2 μM; □, 5 μM; ■, 10 μM; ▲, 20 μM; and ○ 40 μM. Points are experimental values; lines are derived by assuming that eq 2 describes the kinetic mechanism and using the parameters summarized in Table 3.

three diagnostic plots of inhibition type (data not shown), the inhibition by 2′5′-ADP was found to be competitive for the WT enzyme with all the cofactors used. With mutated CAD2, for which the level of the inhibition was lower (for NADPH, the K_i was increased 2.5-fold), it became undependable to assess the inhibition type from these plots. The value of K_i with $NAD(P)^+$ could not be calculated due to the high K_m and the weak inhibition observed.

Table 2: Kinetic Parameters at 30 °C for Both Senses of the Reaction[a]

	WT		mutant S212D		parameter ratio, WT/S212D	
parameter[b]	$NADP^+$ or NADPH	NAD^+ or NADH	$NADP^+$ or NADPH	NAD^+ or NADH	$NADP^+$ or NADPH	NAD^+ or NADH
K_m $NAD(P)^+$ (μM)	3.8 ± 3.1	1230 ± 360	347 ± 113	2120 ± 1220	0.011	0.58
K_m alcohol (μM)	1.4 ± 0.5	241 ± 44	24.7 ± 10.6	193 ± 27	0.060	1.25
K_m NADPH (μM)	5.8 ± 1.5	n.d.	158 ± 32	n.d.	0.037	
k_{cat} $NAD(P)^+$ (s^{-1})	3.33 ± 0.17	1.45 ± 0.08	2.28 ± 0.28	1.90 ± 0.50	1.46	0.76
k_{cat}/K_m $NAD(P)^+$ (μM^{-1} s^{-1})	0.873 ± 0.04	0.0016 ± 0.0007	0.00667 ± 0.0017	0.000833 ± 0.00033	131	1.40
k_{cat} NAD(P)H (s^{-1})	29.4 ± 1.5	n.d.	32.0 ± 1.6	n.d.	0.92	
k_{cat}/K_m alcohol[c]	1.42×10^8	3.6×10^5	5.55×10^6	5.91×10^5	25.6	0.61
k_{cat}/K_m NAD(P)H (μM^{-1} s^{-1})	5.20 ± 1.32	n.d.	0.207 ± 0.042	n.d.	25.2	
overall catalytic efficiency[d] (M^{-2} s^{-1})	6.20×10^{11}	4.9×10^6	2.66×10^8	n.d.	2331	
K_A (A = coniferyl alcohol) (μM	6.97[e]		18.8[f]			0.37
K_B (B = $NAD(P)^+$ or NAD(P)H)(μM	45.7		645		0.07	

[a] In the forward direction the medium was at pH 6.25 for the determination of K_m values for $NAD(P)^+$, and the coniferaldehyde concentration was 50 μM. In the other direction, the medium was at pH 8.8 with the coniferyl alcohol concentration set at 100 μM for the determination of the K_m values for NAD(P)H. In both senses of the reaction, the cofactor concentration was 0.2 mM for the determination of the K_m value of the second substrate (n.d. = not determined). [b] K_m $NAD(P)^+$ means the K_m value for NAD^+ or $NADP^+$ as indicated by the appropriate column heading. Each column indicates $NADP^+$ or NADPH as appropriate for the substrate. Similarly, K_m alcohol means K_m for coniferyl alcohol with the coenzyme used as indicated in the column heading. [c] k_{cat}/K_m refers to $NADP^+$-dependent oxidation of coniferyl alcohol. [d] Overall catalytic efficiency = k_{cat} (alcohol)/($K_m^{alcohol}K_m^{NAD(P)^+}$). [e] For WT CAD, α = 0.25, calculated after Segel, (1975, p 279), with coniferyl alcohol as substrate. [f] For S212D CAD, α = 0.45.

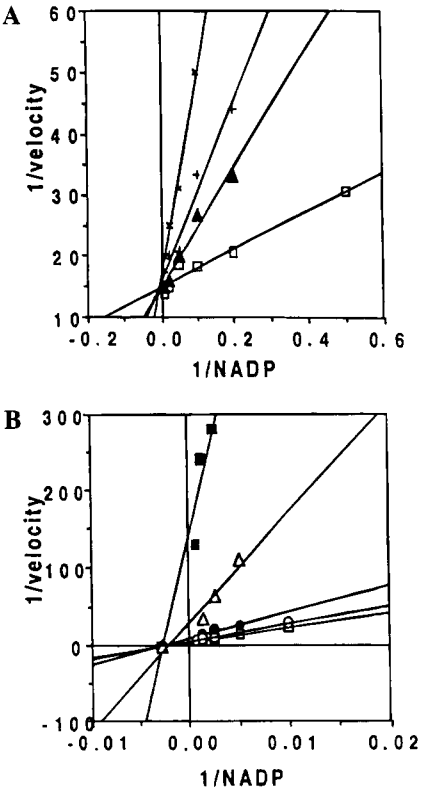


FIGURE 4: Product inhibition plots for CAD with coniferaldehyde as product and with the concentration of $NADP^+$ varied for recombinant WT cinnamyl alcohol dehydrogenase (A) and the S212D mutant enzyme (B), measured at 30 °C, pH 8.8, and at a fixed concentration of coniferyl alcohol of 0.1 mM. The concentrations of coniferaldehyde used were (□) 0, ▲ 5, (+) 10, (○) 15, (×) 20, ● 25, △ 30, and ■ 50 μM.

Substrate Inhibition. From plots of initial velocity for recombinant CAD2 *versus* [coniferyl alcohol] (Figure 5A) and, for the reverse direction, a plot of initial velocity *versus* [coniferaldehyde] (Figure 5B), it is clear that working at 200 μM coniferyl alcohol or 100 μM coniferaldehyde gives velocities which are 73% and 25%, respectively, of the maximal velocity *achievable* under these conditions. For coniferaldehyde as substrate, increasing its concentration by only 2-fold diminishes the observed velocity to 2.7% of maximal. For the reverse direction substrate inhibition is less severe, and increasing the alcohol concentration by 2- and 3-fold decreases the observed assay velocity to 55% and 50% of maximal, respectively.

Substrate inhibition for enzymes such as CAD2 which have complex bisubstrate kinetics is common (Segel, 1975), but there are implications for workers measuring or staining for CAD activity in plants or plant tissues. Working at commonly employed CAD substrate concentrations with a previously unstudied plant or tissue may mean that the substrate level chosen arbitrarily in this way actually lies in a range which shows strong substrate inhibition, and hence an artificially low assay velocity. Lack of detection of CAD activity in a crude extract may reflect particularly strong substrate inhibition in that tissue. As polymorphism has been reported for CAD, substrate inhibition curves may be different from one form to another. This polymorphism exists between plants and also may exist within the same plant at the tissue level since several isoforms have been reported in soybean (Wyrambik & Grisebach, 1975), in bean (Grima-Pettenati et al., 1994), in wheat (Pillonel et al., 1992), and in *Eucalyptus* (Goffner et al., 1992; Hawkins & Boudet,

Table 3: Values of I_{50} for Adenosine 5′-Monophosphate (5′-AMP) Measured for Coniferyl Alcohol Oxidation with 200 μM $NAD(P)^+$ and 0.1 mM Coniferyl Alcohol[a]

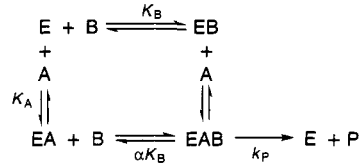
	cofactor	CAD	S212D	$\left(\frac{S212D}{WTCAD}\right)$
I_{50} (μM, 5′-AMP)	$NADP^+$	1580	5000	3.2
	NAD^+	740	2000	2.7
K_i (μM) 2′,5′-ADP	$NADP^+$	100	n.d.	
	NAD^+	11	n.d.	
	NADPH	450	1120	2.5

[a] Also given are values of K_i for adenosine 2′,5′-diphosphate (2′,5′-ADP) for both directions of the enzymatic reaction with 200 μM cofactor and 0.1 mM substrate. n.d., not determined.

1994). Due to the polymorphism of CAD, it is not safe to assay activity using standard conditions and substrate concentrations, as these have to be determined for each tissue and each plant. To ensure that lack of detectable CAD activity is not due to substrate inhibition, we propose that the assay for CAD be conducted at the standard alcohol and aldehyde concentrations used (see Materials and Methods) and also at *half* of those concentrations. If the observed velocity is *increased* by this decrease in substrate concentration, then further substrate dilutions should be made to determine the optimal substrate concentration for that tissue.

DISCUSSION

Overview of the Kinetic Mechanism. WT CAD2 shows a 14-fold (specific activity) preference for $NADP^+$ over NAD^+ and a 28.6-fold preference for NADPH over NADH (Table 1). For the S212D mutation these are reduced to 4.3- and 16.2-fold, respectively. Steady-state kinetic data (Table 2 and Figure 3A−D) support rapid equilibrium random bireactant kinetics for WT and S212D CAD2 [pp 274−279 of Segel (1975)] with the interaction factor $\alpha < 1$, eq 2, confirmed by product inhibition plots (Figure 4) [pp 288 of Segel (1975)].



(2)

For WT CAD,
$\alpha = 0.25$
$K_A = 6.97$ μM (A = coniferyl alcohol)
$K_B = 45.7$ μM (B = $NADP^+$)

For S212D CAD,
$\alpha = 0.45$
$K_A = 18.8$ μM
$K_B = 645$ μM

CAD from soybean (Wyrambik & Grisebach, 1979) showed an ordered bi-bi mechanism, and this may be a species or isozyme difference. The kinetic differences between the CAD enzymes of spruce and soybean (Lüderitz & Grisebach, 1981) may lie in the detailed balance of kinetic constants (or even the mechanism) from complex kinetic models such as eq 2, or in complex parameters such as k_{cat} and K_m reflecting different balances of their component rate constants. Retention of mechanism between WT and mutant allows direct comparison of effects of mutation on kinetic parameters. Mutation D201G in yeast ADH altered the kinetic mechanism (Ganzhorn et al., 1987; Fan et al., 1991). Multiple mutation of glyceraldehyde 3-phosphate dehydrogenase to switch coenzyme specificity led to a change in rate-determining step (Clermont et al., 1993; Corbier et al., 1990).

Considering k_{cat}/K_m values for WT and S212D enzymes, the mutation decreases the rate by 25-fold with NADPH and 131-fold with $NADP^+$ (mostly K_m). This single change is deleterious to the use of NADP(H) by CAD2, in line with the postulated repulsive effect of the negative aspartate side chain on the phosphate anion of NADP(H) and with the slightly increased steric demands of Asp over Ser. Consistent with this, when NAD^+ is coenzyme, the effect of S212D mutation on k_{cat}/K_m is very much less (1.40-fold), with k_{cat} and K_m values being rather similarly affected by the introduction of D212. The effect is not localized totally to the $NADP^+$ site, as k_{cat}/K_m for coniferyl alcohol as substrate

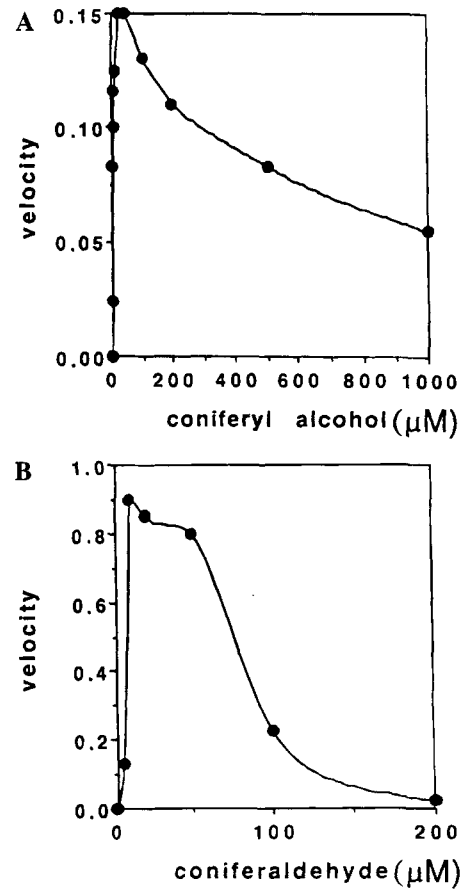


FIGURE 5: Substrate inhibition of WT CAD2 by coniferyl alcohol (A) and coniferaldehyde (B). Plots of initial velocity are shown *versus* the concentration of coniferaldehyde at pH 6.25 (Figure 4B). The concentration of NADP(H) was 200 μM.

(with $NADP^+$ as fixed cofactor) is decreased 25.6-fold in WT relative to S212D. This provides evidence of physical transmission of structural information between the mutation site deep within the $NADP^+$ pocket and the coniferyl alcohol binding region of CAD2. This linkage reflects the tight physical juxtaposition of $NADP^+$ coenzyme (i.e., the electrostatic and steric conflict at the side chain of D212) and enzyme: with NAD^+ there is little effect on k_{cat}/K_m on S212D mutation for the alcohol reaction. In S212D CAD2 using NAD^+, as opposed to $NADP^+$, the electrostatic or steric conflict (see Figure 1) is relieved by the coenzyme's lack of a phosphate ion near the D212 side chain. In line with these physical arguments, α (the degree of interaction between the sites for A and B) is greater for S212D than for WT CAD2. A possibly similar effect has been noted for malate dehydrogenase from *Thermus flavus* (Nishiyama et al., 1993).

The change in binding energy for removal of the 2′-phosphate of $NADP^+$ in the absence of D212 (i.e., WT CAD2) is given (Sem & Kasper, 1993) by eq 3:

$$\Delta\Delta G^{\ddagger} = RT \ln \left(\frac{\left(\frac{k_{cat}}{K_m}\right)_{NADP^+}}{\left(\frac{k_{cat}}{K_m}\right)_{NAD^+}} \right) = -3.9 \text{ kcal/mol} \quad (3)$$

The change in binding energy for 2′-phosphate removal in the presence of D212 (i.e., S212D CAD2) is analogously found to be −1.2 kcal/mol. Thus, the net difference in binding energies for the D212 and S212 side chains with

respect to the 2′-phosphate on the coenzyme is 2.7 kcal/mol. Without considering the steric difference between the side chains of Asp and Ser, this difference is of approximately the same magnitude as the binding energies for the arginine−2′-phosphate interaction in NADPH−cytochrome P450 reductase (Clermont et al., 1993) and for other salt bridges (Fersht, 1972; Santi & Pena, 1973; Stone et al., 1991).

The value of 2.7 kcal/mol cannot be the true energy of the repulsion that the 2′-phosphate experiences, as the CAD2 system displays significant interaction between the alcohol and NAD(P)$^+$ sites. The binding energy difference, $\Delta\Delta G^{\ddagger}$ *toward coniferyl alcohol* between the CAD2 enzymes with serine and aspartate at position 212 for NADP$^+$ and NAD$^+$ as cofactors [$\Delta\Delta G^{\ddagger} = -RT \ln((k_{cat}/K_m)^{WT}/(k_{cat}/K_m)^{S212D})$ using k_{cat}/K_m values for coniferyl alcohol from Table 2] is −1.9 kcal/mol for NADP$^+$, but +0.3 kcal/mol with NAD$^+$, i.e., in the absence of the 2′-phosphate. This energy difference (2.2 kcal/mol), comparable to the change in binding energy for 2′-phosphate removal for the mutant compared to wild type, is substantial. The change in binding energy toward NADPH on replacing D212 by S212 is given by

$$\Delta\Delta G_{D212} = -RT \ln\left(\frac{K_{WT}^{NADPH}}{K_{S212D}^{NADPH}}\right) \quad (4)$$

where K^{NADPH} is the apparent dissociation constant of the indicated enzyme:NADPH complex. Using K_B values (eq 2) gives $\Delta\Delta G$ = 1.6kcal/mol. We were unable to obtain K_A and K_B NAD(H) because of the high K_m for NADH.

Structural Effects of S212D Mutation. With NAD$^+$, little effect (2-fold) is caused by the S212D mutation on K_m, and k_{cat} only changes 1.3-fold. Thus, D212 (mutant) and S212 (WT) interact specifically with the 2′-phosphate (Sem & Kasper, 1993). Using Sem and Kasper's line of argument for CAD2, the small effect (mutant is 0.80 of WT) of mutation on K_m for coniferyl alcohol (NADPH substrate), which binds to CAD2 at a separate, though not *very* distant, site from NADPH (McKie et al., 1993), argues that the mutation does not cause a global structural disruption. The differential effect toward NADP$^+$ (K_m changed 91-fold) *versus* NAD$^+$ (K_m changed only 1.7-fold) seen on S212D mutation is strong evidence that introduction of Asp212 perturbs interaction in the immediate vicinity of the 2′-phosphate bound but has little effect on the binding interactions used by the rest of the NAD(P)$^+$ molecule (proximal effects). Additional evidence against a global structural disruption caused by the mutation is that the kinetic mechanism followed by the mutant CAD2 is the same as that for WT.

As it is not yet clear if K_m is a reasonable measure of cofactor binding strength for cinnamyl alcohol dehydrogenase, the 91-fold increase in K_m for NADP$^+$ on S212D mutation (Table 2) cannot be safely ascribed to an effect primarily on binding; but k_{cat} (NADP$^+$) decreases 1.5-fold on mutation (Table 2), and k_{cat} increases by 1.09-fold for NADPH, with K_m for NADPH increasing 27-fold.

Binding of Inhibitors (5′-AMP and 2′,5′-ADP). While 2′,5′-ADP was found to be a competitive inhibitor of WT enzyme, its inhibition of S212D CAD2 was too weak to allow its unambiguous use as a dead-end inhibitor and obviated its use in affinity chromatography for S212D CAD2. Inhibition by 5′-AMP was also weak, and only I_{50} values are thus reported (Table 3). The weakness of inhibition by 2′,5′-ADP and 5′-AMP must be due to the absence of the binding components of the nicotinamide ring and its β-glycosidically linked 5′-phosphoribose moiety. In gross terms relative to NADP$^+$, 2′,5′-ADP has only half the functionality and molecular volume, and while the detailed weak binding interactions likely to be made by the two parts of the NADP$^+$ molecule to CAD2 [considering the homology model (McKie et al., 1993)] are likely to differ, about half as many weak binding interactions are likely to occur for 2′,5′-ADP as for NADP(H) with CAD2. With K_m as an upper limit to cofactor binding strength, a crude estimate of K_i for 2′,5′-ADP is the square root of K_m for NADP$^+$ (3.8 μM) or NADPH (5.8 μM), estimated at $\leq 2 \times 10^{-3}$M (using 4.8 μM as an average K_m). The observed values of K_i for 2′,5′-ADP are 0.1 mM (*versus* NADP$^+$) and 0.45 mM (*versus* NADPH). This agreement is at first sight consistent with the crude model that 2′,5′-ADP binds in a pocket corresponding to that used by its structurally analogous moiety in NADP$^+$. However, the situation is more complex, as the magnitude of the effect of the S212D mutation on K_i and I_{50} values is approximately the same for NADP$^+$, NADPH, and NAD$^+$ (Table 3). Given the effects of the mutation on substrate parameters (Table 2), this may indicate that 2′,5′-ADP does not bind in the region of the active site occupied by the adenosine phosphate of NADP$^+$ or NADPH. From the approximate internal symmetry of the NAD(P)$^+$ molecule, taking adenine and nicotinamide as roughly equivalent, 2′,5′-ADP may inhibit by binding to the region of the CAD2 active site occupied by the nicotinamide phosphoribotide portion of NADP(H). This situation was modeled by superimposing the furanose ring oxygen, C1′, C3′, and 5′-phosphate phosphorus atoms of 2′,5′-ADP and the nicotinamidofuranose ring of NADPH in the CAD2 homology model active site. When this was done, the adenine ring of 2′,5′-ADP was found to lie in the cavity occupied by the nicotinamide ring of NADPH with no steric conflicts. The sugar rings of the two ligands having been superimposed presented no clashes with CAD2, and the 5′-phosphate group of 2′,5′-ADP was located in the region occupied by the 5′-phosphate of the nicotinamidoribose. The 2′-phosphate group of 2′,5′-ADP was located by this protocol in a pocket bounded by His52 and His48, which with minor side-chain rotation could provide hydrogen-bonding and/or electrostatic stabilization (depending on the pK_a of these residues when 2′,5′-ADP is bound). While 2′,5′-ADP could in principle serve as a dead-end inhibitor of such dehydrogenases, its detailed binding mechanism may be very different from that of the ligand for which it is used as an analogue.

Overall Catalytic Efficiency. The overall catalytic efficiency of dehydrogenases is defined as $k_{cat}/(K_m^A \times K_mB)$ where K_m^A and K_m^B are the K_m values for substrates A and B, respectively (Feeney et al., 1990). The overall catalytic efficiency for the NADP$^+$ direction (Table 3) decreases 2.33 $\times 10^3$-fold for the S212D mutation with NADP$^+$, but not for NAD. For WT CAD2, the decrease in catalytic efficiency using NAD$^+$ rather than NADP$^+$ is 1.27×10^5. For S212D CAD2 cofactor discrimination is much less (57.8-fold in favor of NADP$^+$ relative to NAD$^+$). Overall the cofactor preference for NADP$^+$ over NAD$^+$ decreased 2.2×10^3-fold (=1.27×10^5/47.8) by a single mutation.

Comparison with Coenzyme Switching Studies for Other Dehydrogenases and Reductases. The most effective switches

in coenzyme specificities have come from multiple mutations (Scrutton et al., 1990; Mittl et al., 1993, 1994; Bocanegra et al., 1993). For *Drosophila* ADH, the D39N mutant hardly affected the value of k_{cat}/K_m for NAD^+ but increased it 589-fold for $NADP^+$ (Chen et al., 1991, 1994). The corollary of the present study on *Eucalyptus* CAD2 is the D53 mutation for NAD-dependent lactate dehydrogenase (Feeney et al., 1990), which had only a small effect on kinetic parameters for NADH or NADPH. Using overall catalytic efficiency (Feeney et al., 1990), D53S mutation of lactate dehydrogenase shifted the enzyme 20-fold toward NADPH. The reverse-sense mutation of the present study (S212D) for CAD2 has more marked effects (overall catalytic efficiency decreases 2.2×10^3-fold when NAD^+ is used rather than $NADP^+$). Focusing on k_{cat}/K_m values for NADPH with WT CAD (5.2×10^6 M^{-1} s^{-1}), compared to NADH with S212D CAD (0.207×10^6 M^{-1} s^{-1}), shows that the single mutation allows NADH to operate as coenzyme for the mutant enzyme at 1/25 the rate of NADPH with WT. Thus CAD2 provides a good basis for any future multiple-mutation engineered switch in coenzyme specificity. Coenzyme-switched forms of enzymes have been proposed (Perham, 1991) as potential metabolic and physiological probes of the effects of altered coenzyme usage. CAD2 may provide the basis of such a tool in plant biochemistry and physiology, as it appears to be crucial to lignification.

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