16,17-Dihydro Gibberellin A_5 Competitively Inhibits a Recombinant Arabidopsis GA 3β-Hydroxylase Encoded by the *GA4* Gene

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Ring D-modified gibberellin (GA) A_5 and A_{20} derivatives are structurally similar to GA_{20} and GA_9 (the precursors to growth-active GA_1 and GA_4) and, when applied to higher plants, especially grasses, can reduce shoot growth with concomitant reductions in levels of growth-active GAs and increases in levels of their immediate 3-deoxy precursors. The recombinant Arabidopsis GA 3β-hydroxylase (AtGA3ox1) protein was used in vitro to test a number of ring D-modified GA structures as possible inhibitors of AtGA3ox1. This fusion protein was able to 3β-hydroxylate the 3-deoxy GAs, GA_9 and GA_{20}, to GA_4 and GA_1, respectively, and convert the 2,3-didehydro GA, GA_5, to its 2,3-epoxide, GA_6. Michaelis-Menten constant (K_m) values of 1.25 and 10 μM, respectively, were obtained for the GA_9 and GA_{20} conversions. We utilized the enzyme's ability to convert GA_{20} to GA_1 in order to test the efficacy of GA_5, 16,17-dihydro GA_5 (dihydro GA_5), and a number of other ring D-modified GAs as inhibitors of AtGA3ox activity. For the *exo*-isomer of dihydro GA_5, inhibition increased with the dose of dihydro GA_5, with Lineweaver-Burk plots showing that dihydro GA_5 changed only the K_m of the enzyme reaction, not the V_{max}, giving a dissociation constant of the enzyme-inhibitor complex (K_i) of 70 μM. Other ring D-modified GA derivatives showed similar inhibitory effects on GA_1 production, with 16,17-dihydro GA_{20}-13-acetate being the most effective inhibitor. This behavior is consistent with dihydro GA_5, at least, functioning as a competitive substrate inhibitor of AtGA3ox1. Finally, the recombinant AtGA3ox1 fusion protein may be a useful screening tool for other effective 3β-hydroxylase inhibitors, including naturally occurring ones.

We now know, through the use of gibberellin (GA) biosynthesis mutants (for review, see Reid and Howell, 1995; Hedden and Proebsting, 1999), that GA_1, GA_4, and other 3β-hydroxylated GAs are active per se in stem elongation, while their immediate 3-deoxy precursors, i.e. GA_{20} and GA_9, are not. The biosynthetic conversions of GA_{20} and GA_9 to GA_1 and GA_4, respectively, are catalyzed by GA 3β-hydroxylases (Lester et al., 1997; Martin et al., 1997; Williams et al., 1998; Itoh et al., 2001). 16,17-Dihydro GA_5 (Fig. 1) and other ring D-modified GA_5 derivatives (Mander et al., 1995, 1998a, 1998b) are structurally very similar to GA_{20}.When these ring D-modified GAs are applied to many higher plant species, and especially to grasses, they can effectively inhibit shoot growth (Evans et al., 1994a, 1994b; Takagi et al., 1994; Foster et al., 1997; King et al., 1997, 2004). Associated with the reduction in shoot elongation is a concomitant reduction in levels of endogenous 3β-hydroxylated GAs and an increase in one or more of the 3-deoxy precursors (Foster et al., 1997; Junttila et al., 1997; Zhou, 2000). For example, application of *exo*-16,17-dihydro GA_5-13-acetate to wild oat reduces levels of endogenous GA_1, GA_3, and GA_4 while elevating levels of their immediate 3-deoxy precursors, GA_9 and GA_{20} (Zhou, 2000). It thus appears likely that 16,17-dihydro GA_5 and allied ring D-modified GA derivatives gain their efficacy as growth-retarding compounds in grasses (Evans et al., 1994a; Mander et al., 1995; Foster et al., 1997; Zhou, 2000) by inhibiting per se the action of the GA 3β-hydroxylase(s) which catalyze(s) the conversion of GA_{20} to GA_1 and GA_9 to GA_4, respectively.

To examine this possibility, the Arabidopsis GA 3β-hydroxylase (AtGA3ox1) encoded by the *GA4* gene was expressed in *Escherichia coli*. We then used this recombinant protein for a series of in vitro tests, both as the crude lysate (Zhou et al., 1998) and as a purified fusion protein (Zhou, 2000), to examine the ability of a range of concentrations of GA_5, the *endo*- and *exo*-isomers of 16,17-dihydro GA_5 and 16,17-dihydro GA_{20}, three 13-O-acetyl derivatives of these two dihydro GAs, 16,17-methano-dihydro GA_5 and 16,17-dichloromethano-dihydro GA_5, to inhibit GA 3β-hydroxylase activity (i.e. inhibit conversion of GA_{20} to GA_1 by AtGA3ox1). A parallel approach has also been taken by King et al. (2004) using cell lysates containing recombinant 3β- and 2β-hydroxylases from *Pisum sativum*, where their range of ring D-modified GA_5 derivatives included the 17-ethyl, *n*-propyl, and *n*-butyl derivatives of dihydro GA_5.

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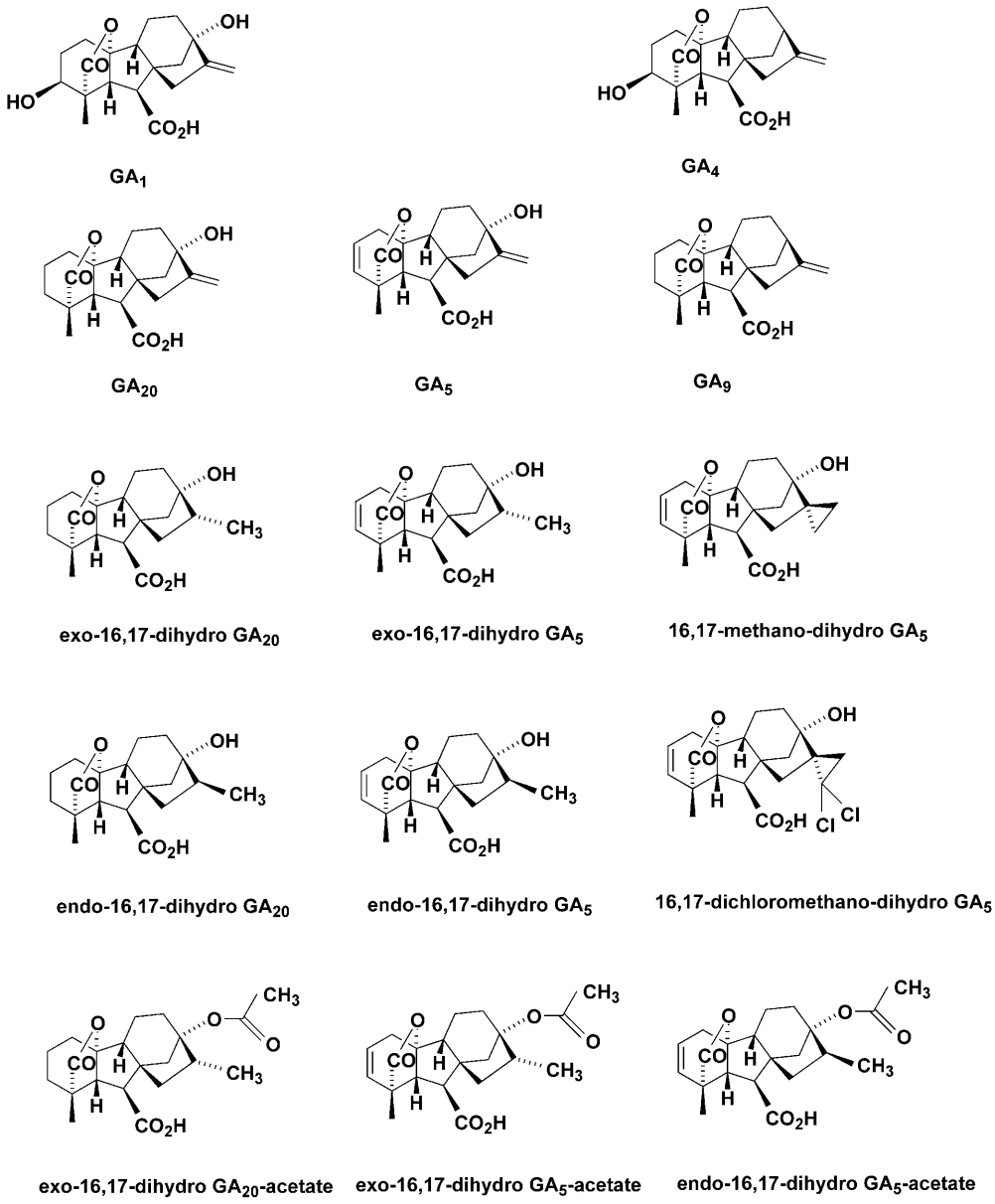

Figure 1. Structures of GA_1, GA_4, GA_9, GA_{20}, GA_5, and several ring D-modified GA derivatives.

RESULTS

Williams et al. (1998) showed that AtGA3ox1, which is encoded by the *GA4* gene, was a GA 3β-hydroxylase. To examine the inhibitory effects of ring D-modified GA derivatives, we also expressed this enzyme as a fusion protein in *E. coli*. Both the crude *E. coli* lysate and purified fusion protein, with the addition of appropriate cofactors, effectively 3β-hydroxylated 3-deoxy GAs (e.g. GA_{20} or GA_9). However, in this paper we present only results obtained with the purified fusion protein.

Characterization of Bacterially Expressed AtGA3ox1

For longer term reactions, the fusion protein's ability to 3β-hydroxylate GA_{20} to GA_1 and GA_5 to GA_6 was quite sensitive to pH, with enzyme activity being reduced appreciably below pH 6 or above pH 9. The optimal pH, then, for AtGA3ox1 activity, was determined to be 7.5, a finding similar to that obtained by Williams et al. (1998). When the AtGA3ox1 fusion protein was diluted in assay buffer containing essential cofactors, its activity at room temperature increased gradually up to hour 1 (Fig. 2). However, by hour 2, enzyme activity had dropped by nearly 50%. All subsequent assays were thus run with enzyme that had been preincubated at room temperature for 30 to 40 min. The identity and amounts of reaction products, GA_4 and GA_1, were determined by gas chromatography-mass spectrometry-selected ion monitoring (GC-MS-SIM) as the methyl ester trimethylsilyl ether (MeTMSi) derivatives, with quantification being accomplished through stable isotope dilution after

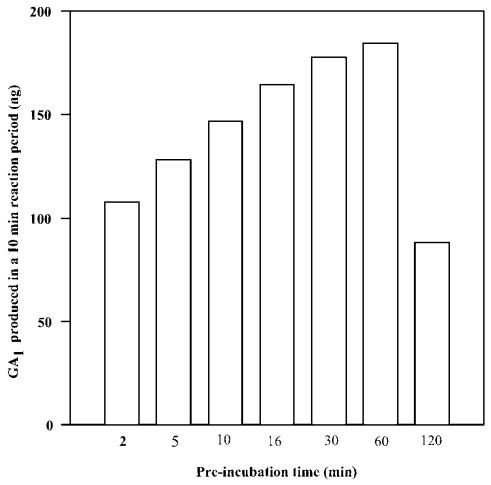

Figure 2. Stability of recombinant AtGA3ox1 at room temperature. Enzyme (0.02 μg protein) was diluted in 80 μL Tris-HCl buffer, pH 7.5 containing 0.1 mM Fe^{2+}, 5 mM ascorbate, 5 mM 2-oxoglutarate, 2 mM NADPH, and 0.2 mg BSA. This mixture was incubated at room temperature for varying periods before being mixed with 20 μL of a 50 μM solution of GA_{20} to form a reaction mixture. Quantification of GA_1 was accomplished with the isotope dilution method using GC-MS-SIM (a known amount of $[^2H_2]GA_1$ was added just prior to extracting the reaction mixture with EtOAc).

addition of known amounts of $[^2H_2]GA_4$ and $[^2H_2]GA_1$ (Fujioka et al., 1988). Plots of the reciprocal of reaction rate against the reciprocal of substrate concentration were produced by linear regression analysis (Fig. 3, A and B). These yielded K_m values of 1.25 μM (V_{max} 800 nmol min^{-1} mg^{-1} protein) for GA_9 and 10 μM (V_{max} 62 nmol min^{-1} mg^{-1} protein) for GA_{20}, respectively, values similar to those reported by Williams et al. (1998).

Mode of Action of 16,17-Dihydro GA_5 in Inhibiting AtGA3ox1 Activity

We initially tested the dose effects of four ring D-modified GA derivatives on the ability of AtGA3ox1 to produce GA_1 from GA_{20} (Fig. 4). Enzyme activity decreased in a linear fashion as the log dose of *exo*-16,17-dihydro GA_5 increased (Fig. 4B). A similar response occurred for *endo*-16,17-dihydro GA_5, *endo*-16,17-dihydro GA_{20}, and *exo*-16,17-dihydro GA_{20} (Fig. 4, A, C, and D, respectively). Then we utilized Michaelis-Menten plots to examine the effect of a 100 μM dose of the *exo*-isomer of dihydro GA_5 on AtGA3ox1 activity (reaction velocity) across a range of GA_{20} concentrations (Fig. 3B, black circles). The V_{max} in the presence of the dihydro GA_5 remained the same as for GA_{20} alone, even though the slope increased (Fig. 3B). Hence, only the K_m of the reaction was changed, not the V_{max}. The dihydro GA_5 molecule is thus functioning as a competitive substrate inhibitor in the AtGA3ox1 enzyme's conversion of GA_{20} to GA_1. The dissociation constant of the enzyme-inhibitor complex (K_i) for *exo*-16,17-dihydro GA_5 was calculated to be 70 μM (Fig. 3B).

We then tested the inhibitory effects of 16,17-dihydro GA_5, 16,17-dihydro GA_{20}, and a number of other ring D-modified GA_5 derivatives, as well as GA_5, all at 100 μM, on 3β-hydroxylation of 10 μM of GA_{20} by the fusion protein (Table I). Thus, when added to GA_{20}, the aliquots of GA_5, *exo*-16,17-dihydro GA_5 and *endo*-16,17-dihydro GA_5, all reduced GA_1 production by nearly 50%. 16,17-Methano-dihydro GA_5 and 16,17-dichloromethano-dihydro GA_5, when added at 10-fold the concentration of GA_{20}, reduced GA_1 production by 70% (Table I). The most effective of the dihydro GAs, however, was dihydro GA_{20}, where both the *endo*- and *exo*-isomers (Fig. 4, C and D), when

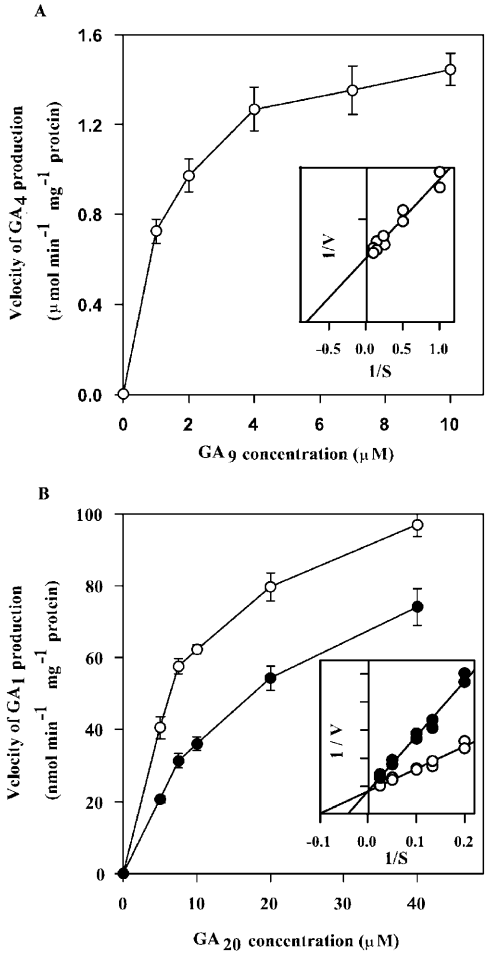

Figure 3. Michaelis-Menten and Lineweaver-Burk (inset) plots for AtGA3ox1's conversion of GA_9 to GA_4 (A) and GA_{20} to GA_1 (B). Enzyme (0.01 μg for A and 0.1 μg for B) was diluted in 90 μL Tris-HCl buffer, pH 7.5 containing 0.1 mM Fe^{2+}, 5 mM ascorbate, 5 mM 2-oxoglutarate, 2 mM NADPH, and 0.2 mg BSA. This mixture was incubated at room temperature for 30 min before adding GA_9 or GA_{20}, with (black circles) or without (white circles) the addition of *exo*-16,17-dihydro GA_5 at a final concentration of 100 μM to form a reaction mixture. The reaction period was 5 min. Quantification of GA_4 and GA_1 was accomplished by the isotope dilution method using GC-MS-SIM (a known amount of $[^2H_2]GA_4$ or $[^2H_2]GA_1$ was added to the reaction mixture just before extraction with EtOAc).

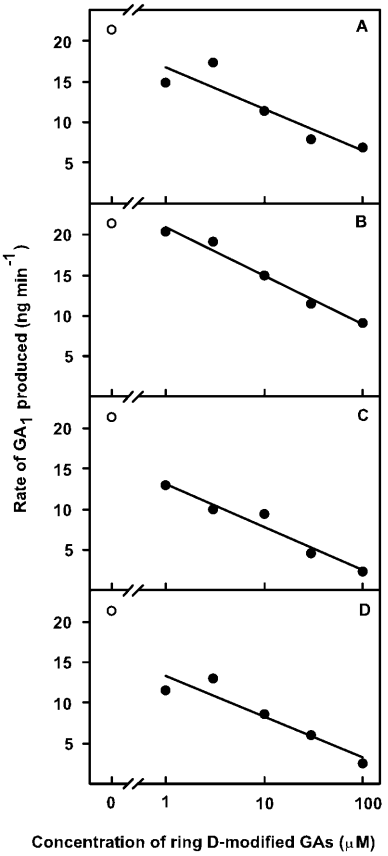

Figure 4. Effects of varying the dose (1, 3.3, 10, 33, and 100 μM) of ring D-modified GA derivatives on the production of GA_1 from substrate GA_{20} in the presence of AtGA3ox1. The same amount of enzyme was added to each reaction mixture. The initial GA_{20} concentration was 10 μM. Cofactors added included 0.1 mM Fe^{2+}, 5 mM ascorbate, 5 mM 2-oxoglutarate, 2 mM NADPH, and 2 mg BSA mL^{-1}. The control is 10 μM GA_{20} alone, plus cofactors (white circle, upper left). A, *endo*-dihydro GA_5 (r^2 = 0.98); B, *exo*-dihydro GA_5 (r^2 = 0.82); C, *endo*-dihydro GA_{20} (r^2 = 0.95); D, *exo*-dihydro GA_{20} (r^2 = 0.89). The reaction period was 10 min. Quantification of GA_1 was accomplished by GC-MS-SIM using a known amount of $[^2H_2]GA_1$ which was added after stopping the reaction mixture, just before extraction with EtOAc.

added at 10-fold the GA_{20} concentration, reduced GA_1 production by 79% (Table I).

Interestingly, addition of an acetate group at C-13 increased inhibition efficacy by approximately 10-fold, with the *exo*-isomers (most active) of each of dihydro GA_5-Ac and dihydro GA_{20}-Ac reducing GA_1 formation by 96.4% and 99.7%, respectively (Table I).

Thus, GA_5 and all of the ring D-modified GA_5 or GA_{20} derivatives inhibited the conversion of GA_{20} to GA_1 by AtGA3ox1, with *exo*-16,17-dihydro GA_{20}-13-O-acetate being the most effective (Table I). However, only the *exo*-isomer of 16,17-dihydro GA_5 has actually been shown to function as a competitive substrate inhibitor (Fig. 3).

Also of interest with regard to use of the 13-acetate derivatives of dihydro GA_5 and dihydro GA_{20} is the fact that there was no production of dihydro GA_5 or dihydro GA_{20} during the reaction. This was ascertained by GC-MS-SIM analysis of the reaction mixture. Thus, the *exo*-isomers of each of 16,17-dihydro GA_5-13-O-acetate and 16,17-dihydro GA_{20}-13-O-acetate, the most potent of the ring D-modified GAs, are per se active as inhibitors of the 3β-hydroxylase without conversion to their 13-hydroxyl forms. We also checked the reaction mixtures by GC-MS full scan and SIM for products of 16,17-dihydro GA_5, i.e. dihydro GA_6 or dihydro GA_3, and found no evidence of its metabolism. However, when we checked the reaction mixture containing 16,17-dihydro GA_{20}, putative 16,17-dihydro GA_1 (tetrahydro GA_3) was identified by GC-MS full scan based on its fragmentation pattern and retention time (Rt) relative to the Rt of $[^2H_2]GA_1$ (which was present as an internal standard). Thus, the M^+ (508) and characteristic *m/z* fragmentation ions 465, 450, and 209 for 16,17-dihydro GA_1 were present at relative intensities shown by authentic 16,17-dihydro GA_1. Additionally, the capillary GC Rt of authentic dihydro GA_1, relative to the Rt of $[^2H_2]GA_1$, was consistent with the Rt we found for the putative dihydro GA_1. Based on use of the deuterated GA_1 internal standard, it was apparent that the amount of putative dihydro GA_1 formed increased with the reaction time, at least in the first 10 min.

DISCUSSION

The K_m values of AtGA3ox1 fusion protein (in a pGEX-2T vector) for the conversions of GA_9 to GA_4 and GA_{20} to GA_1 were approximately the same as those obtained by Williams et al. (1998) with lysate preparations and also roughly comparable to those seen for other GA3ox1 enzymes expressed in different vectors (Martin et al., 1997; King et al., 2004). However, purified AtGA3ox1 fusion protein was much more

Table I. *Inhibitory effects of GA_5 and a range of ring D-modified GA_5 and GA_{20} derivatives on 3β-hydroxylation of GA_{20} by recombinant AtGA3ox1*

Initial GA_{20} concentration was 10 μM. Gibberellin A_5 and the various GA derivatives were added at a concentration of 100 μM. Values are the mean of two replicate experiments that tested all compounds on the same day.

	GA_1 Produced
	% of control
GA_{20} alone	100[a]
GA_{20} + GA_5	52.4 ± 1.2
GA_{20} + *exo*-16,17-dihydro GA_5	51.4 ± 5.7
GA_{20} + *endo*-16,17-dihydro GA_5	51.2 ± 0.9
GA_{20} + 16,17-dichloromethano-dihydro GA_5	27.5 ±1.9
GA_{20} + 16,17-methano-dihydro GA_5	30.3 ± 6.0
GA_{20} + *exo*-16,17-dihydro GA_{20}	21.1 ± 2.6
GA_{20} + *endo*-16,17-dihydro GA_{20}	21.9 ± 0.4
GA_{20} + *exo*-16,17-dihydro GA_5-acetate	3.6 ± 0.1
GA_{20} + *endo*-16,17-dihydro GA_5 acetate	6.0 ± 0.3
GA_{20} + *exo*-16,17-dihydro GA_{20}-acetate	0.26 ± 0.01

[a]90 ng of GA_1 was produced in 5 min.

active than the earlier lysate preparations, with a V_{max} of 800 and 62 nmol min^{-1} mg^{-1} protein for GA_9 and GA_{20} at 20°C (Fig. 3). For example, at optimal pH, a 1-μg aliquot of enzyme, incubated for 1 min at room temperature with a 4 μM concentration of GA_9 or 2 μM GA_{20} substrate in a final volume of 100 μL, produced 300 pmol of GA_4 and 22 pmol of GA_1, respectively (data not shown). This contrasts with only 5.5 and 1 pmol of GA_4 and GA_1, respectively, for 1 mg of protein incubated at 30°C for 1 min using 4 μM of GA_9 and GA_{20} as substrate (Williams et al. 1998).

Our lysate preparations appeared to contain AtGA3ox1 inhibitors. That is, when we mixed 3 μL of AtGA3ox1 lysate with 87 μL of control lysate (prepared from bacteria transformed with the empty vector only) in a 100-μL reaction volume, the GA 3β-hydroxylase activity was reduced at least 15-fold (data not shown). Although this type of diminished activity seems most likely to be due to the presence of inhibitors of the hydroxylation reaction, one cannot rule out the possibility that bacterial proteases (which could digest AtGA3ox1) also occur in the lysate preparations. We also found that detergent can reduce the activity of the AtGA3ox1 fusion protein, e.g. Triton X-100 reduced enzyme activity by 75% (data not shown).

The AtGA3ox1 fusion protein is relatively unstable, with activity increasing initially with incubation time up to 1 h, but diminishing after that (Fig. 2). We cannot explain the initial gradual increase of enzyme activity. The AtGA3ox1 fusion protein prefers GA_9 as a substrate, followed by GA_{20} and, finally, GA_5. When similar amounts of GA_9, GA_{20}, and GA_5 were pooled and then incubated with the enzyme, it produced predominantly GA_4, only a small amount of GA_1, and virtually no GA_6 (data not shown). This trend in efficacy can also be seen from a comparison of the K_m or K_i values, i.e. GA_9, 1.25 μM; GA_{20}, 10 μM; and GA_5, 55 μM (the K_m's of GA_9 and GA_{20} were calculated from Fig. 3; the K_i of GA_5 was estimated from Table I using 100 μM of GA_5 incubated with 10 μM of GA_{20}). This finding is in general agreement with earlier reports (Martin et al., 1997; Williams et al., 1998), although Kwak et al. (1988) found that a partially purified GA 3β-hydroxylase from immature seeds of *Phaseolus vulgaris* had a slightly higher affinity for GA_{20} than GA_9. Thus, enzymes isolated from different plant species or even different tissues of the same plant may or may not show differences in substrate preference.

It is common that compounds with similar structures act as substrate competitors in biosynthetic steps. For the GA 3β-hydroxylase, we would expect that a range of GA structures might compete for the active site(s) with the preferred substrates, GA_9 or GA_{20}. Based on evidence in hand for rice (Takagi et al., 1994), sorghum (Foster et al., 1997), Lolium (Junttila et al., 1997), wild oat (Zhou, 2000), or a wild-type pea genotype (King et al., 2004), this type of competition is strongly implied for those ring D-modified GAs that can effectively inhibit shoot growth. Accompanying the inhibition of stem and leaf elongation that was obtained by exogenous application of 16,17-dihydro GA_5 and/or its derivatives to various grass species were lowered levels of GA_1 and/or GA_4 and very elevated levels of GA_{20} and GA_9 (King et al., 2004).

We thus tested the hypothesis that the ring D-modified GAs are acting as competitive substrate inhibitors by using the purified AtGA3ox1 fusion protein in an in vitro system that could rapidly convert GA_{20} to GA_1 (see ''Results''). Using the *exo*-isomer of 16,17-dihydro GA_5 (Fig. 1) and expressing the results in a Lineweaver-Burk plot of enzyme activity, it was apparent that dihydro GA_5 changed only the K_m of the enzyme, with the V_{max} remaining the same (Fig. 3B). Thus, dihydro GA_5 is indeed a competitive substrate inhibitor of AtGA3ox1. In a parallel study with recombinant pea *Le* 3β-hydroxylase, where tritium release from [^{3}H]GA_{20} was utilized as a measure of 3β-hydroxylation, King et al. (2004) provided evidence that implies a similar mechanism (competitive inhibition) for certain, but not all, of the ring D-modified GAs that they tested. However, their data did not allow them to determine whether or not the inhibition was truly competitive for these compounds. For the pea GA3ox, while GA_5 was a fair inhibitor (it reduced 3β-hydroxylation to 40% of control at 100 μM), dihydro GA_5 inexplicably showed very poor activity, although dihydro GA_5-13-Ac was a good inhibitor, i.e. it reduced GA_1 production to 30% of control at 10 μM (King et al., 2004). In contrast, for the Arabidopsis AtGA3ox1, GA_5 and dihydro GA_5 were equally effective, dihydro GA_{20} was very effective, and the 13-acetate derivatives of these two dihydro GAs were exceptionally effective enzyme activity inhibitors (Table I). Hence, ignoring purification differences and other variables, there may well be inherent differences between the Arabidopsis and pea 3β-hydroxylase enzymes in their ability to recognize ring D-modified GAs as competitive substrate inhibitors. The inhibition of GA 3β-hydroxylation by certain GAs (Kwak et al., 1988) and C/D-ring-rearranged GA isomers (Saito et al., 1992), using a partially purified bean GA 3β-hydroxylase, has also been described. It is thus possible that a wide range of GA_5 or GA_{20} derivatives (Table I) will function as competitive substrate inhibitors not only of AtGA3ox1, but also of 3β-hydroxylases from other plant species.

Among the 16,17-dihydro GAs that we tested, *exo*-16,17-dihydro GA_{20}-13-O-acetate was the most effective inhibitor of AtGA3ox1 (Table I). The *exo*-isomer of 16,17-dihydro GA_5-O-13-acetate was also quite effective, with 1.7-fold greater inhibitory activity than the comparable *endo*-isomer (Table I). However, using the simpler structures (e.g. dihydro GA_5 and dihydro GA_{20} which have a C-13 hydroxyl), there were essentially no differences in efficacy between the two exocyclic isomers (Table I).

The K_i value obtained for *exo*-dihydro GA_5 in its inhibition of the GA_{20} to GA_1 conversion is about 70 μM. In contrast, the K_m values for GA_9 and GA_{20} in

their conversions to GA_4 and GA_1, respectively, are 1.25 and 10 μM (as calculated from Fig. 3). Thus, the inhibitory effect of dihydro GA_5 on the in vitro activity of AtGA3ox1 is really quite limited. This can also be seen from results shown in Table I, where inhibitory activity of dihydro GA_5 is compared with a range of ring D-modified GAs, including the highly effective 13-O-acetate- and 16,17-dichloromethano derivatives of dihydro GA_5 and dihydro GA_{20}. Even so, dihydro GA_5 is a very effective inhibitor in situ of both growth and GA_{20} 3β-hydroxylation in rice (Takagi et al., 1994), sorghum (Foster et al., 1997), Lolium (Junttila et al., 1997), and wild oat (Zhou, 2000).

Although GA_5 and dihydro GA_5 give similar inhibitory effects (Table I), GA_5 stimulates stem elongation while dihydro GA_5 usually inhibits it, especially in grasses (Evans et al., 1994b). The growth-promotive activity of GA_5 can be explained in a number of ways. For example, GA_5 may be per se active (as postulated by Spray et al. [1996]), or else growth-active metabolites of GA_5 (e.g. GA_3, GA_6) are produced (Fujioka et al., 1990). We think that the latter hypothesis is more likely. This conclusion is based on in situ experiments that showed production of growth-active metabolites (see above), together with in vitro enzyme assays using AtGA3ox1 (GA_5 converted to GA_6; Williams et al., 1998, and herein) and OsGA3ox1 (GA_5 converted to GA_3; Itoh et al., 2001). That said, another explanation for the activity of GA_5 is the possibility that GA_5 may block GA_{20} 2β-hydroxylation (which yields GA_{29}, an inactive catabolite), an effect that would place appreciably increased amounts of GA_{20} into the biosynthetic pathway where it could be converted to growth-active GA_1. Indeed, such an effect was seen for GA_5 when it was used in vitro to inhibit the action of recombinant pea GA2ox1 enzyme (which catalyzes the GA_{20} to GA_{29} step; King et al., 2004).

Different dihydro GA_5 derivatives have very different inhibitory effects on AtGA3ox1 activity, thus implicating structural differences between the derivatives at C-3, C-13, and C-16,17 as the causal factor(s). Relative to GA_{20}, the C-2,3 double bond of GA_5 reduces, by fivefold, the affinity of AtGA3ox1 for the GA molecule (Table I). In contrast, relative to GA_5, the loss of the double bond at C-16,17, i.e. dihydro GA_5, did not increase inhibition of AtGA3ox1's activity (Table I). However, it was somewhat surprising to note that for pea GA3ox1, dihydro GA_5 was very much less effective as an inhibitor than GA_5 (King et al., 2004). When one makes further modifications at C-16,17, a very enhanced inhibitory effect on activity of AtGA3ox1 can be obtained. For example, relative to 16,17-dihydro GA_5, both 16,17-methano-dihydro GA_5 and 16,17-dichloromethano-dihydro GA_5 have much greater inhibitory effects on AtGA3ox1 activity (Table I). In a somewhat similar fashion, modifying the exocyclic 17-methyl (dihydro GA_5) to exocyclic ethyl, *n*-propyl and *n*-butyl structures also gave enhanced inhibition of 3β-hydroxylation (relative to dihydro GA_5) for the recombinant pea GA 3β-hydroxylase (King et al., 2004). Finally, replacing the 13-hydroxyl of GA_5 with a 13-O-acetate substantially increased inhibitory activity against the pea GA 3β-hydroxylase (King et al., 2004). Also, replacing the 13-hydroxyl with a hydrogen, i.e. GA_9, gives about a sevenfold higher affinity (relative to GA_{20}) for AtGA3ox1 (Fig. 3; Williams et al., 1998) and a similar relationship occurs even with the pea 3β-hydroxylase (Martin et al., 1997).

Dihydro GA_5-13-O-acetate and especially dihydro GA_{20}-13-O-acetate gave exceptionally enhanced (10- to 100-fold increases) inhibition of AtGA3ox1 activity, relative to the dihydro GA analogs with a free hydroxyl at C-13 (Table I). However, we did not know whether the 13-acetates of 16,17-dihydro GA_{20} and dihydro GA_5 function per se in the living plant, or first undergo hydrolysis of the 13-acetate group. A preliminary experiment with [^{3}H]GA_1-13-acetate in rice (D.W. Pearce, R.P. Pharis, and L.N. Mander, unpublished data) did show a rapid conversion of the 13-acetate to [^{3}H]GA_1. However, our in vitro work with AtGA3ox1 demonstrates that the 13-acetate forms of dihydro GA_5 and dihydro GA_{20} exert their inhibitory effects directly (e.g. GC-MS-SIM of the reaction mixture showed no presence of a 13-hydroxylated dihydro GA [i.e. no dihydro GA_5 or dihydro GA_{20}] after the reaction was finished).

We also found that purified AtGA3ox1 did not produce detectable levels (based on GC-MS full-scan analysis of the reaction mixtures) of any metabolites of dihydro GA_5. We had expected that there might be production of dihydro GA_3 or dihydro GA_6 based on earlier work with a cell-free system from immature bean seeds by Saito (1990) using C/D-rearranged GA_5. King et al. (2004) also reported a finding similar to ours (no metabolism of 16,17-dihydro GA_5) for the pea *Le* GA3ox. Hence, there may be inherent differences between species, or else the crude, cell-free system used by Saito et al. (1992) may have contained essential cofactors that were missing in our reaction mixture or in the reaction mixture of King et al. (2004). We did, however, find that 16,17-dihydro GA_{20} was metabolized in our reaction mixture to putative 16,17-dihydro GA_1 by AtGA3ox1 and that the production of putative dihydro GA_1 increased with time. Of interest here is the likelihood that production of dihydro GA_1 would likely have resulted in a somewhat reduced level of enzyme-inhibitory activity by the dihydro GA_{20} in the reaction mixture (Table I), since dihydro GA_1 has been shown to be growth promotive on stem elongation in Lolium (Evans et al., 1994b). The recombinant purified AtGA3ox1 system is a very useful tool in better understanding the interaction between substrate structure and enzyme function for the 3β-hydroxylation step in GA biosynthesis. Additionally, this system may be useful for screening GA 3β-hydroxylase inhibitors, including naturally produced inhibitors. Finally, the AtGA3ox1 fusion protein can also be used to synthesize stable isotope- or radioisotope-labeled GAs. Such a use of recombinant AtGA3ox1 has recently been described by Tudzynski et al. (2003).

MATERIALS AND METHODS

Expression of Arabidopsis AtGA3ox1 Protein in *Escherichia coli*

A sense primer (5′ to 3′, GGGGATCCATGCCTGCTATGTTAACAG) and an antisense primer (5′ to 3′, GGGGATCCTTCTTCTCTGTGATTTCTAA), with a *Bam*HI restriction site for each primer, were designed based on the Arabidopsis *GA4* cDNA sequence (Chiang et al., 1995). When pGEX-2T vector was used, the designed sense primer allowed the GST reading frame to read through the *GA4* insert. PCR was then performed using the Arabidopsis *GA4* cDNA clone pCD7 (a gift from Dr. H.M. Goodman, Harvard Medical School, Boston, MA) as the template. The reaction mixture (100 μL) contained 250 μM dNTPs, 2 mM $MgCl_2$, 40 pmol of each primer, and 20 units of cloned Pfu DNA polymerase (Stratagene, La Jolla, CA). The reaction was heated to 94°C for 2 min, then subjected to 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min. The amplified PCR product was digested with *Bam*HI and ligated into the dephosphorylated *Bam*HI site of the pGEX-2T vector before *E. coli* DH5α cells were transformed. Isolated plasmids were examined with a PCR reaction using the sense and 3′ pGEX sequencing primers to see if they contained the correct insert oriented in the correct direction. The clone with the correct orientation was then sequenced. Transformed *E. coli* were inoculated into 5 mL of an LA broth (1% [w/v] NaCl; 1% [w/v] tryptone; and 0.5% [w/v] yeast extract containing 100 μg amphicillin mL^{-1};) and initially incubated at 37°C overnight with shaking. Then, after the addition of isopropyl-1-thio-α-D-galactopyranoside (IPTG) to a final concentration of 0.2, 0.4, 0.6, and 0.8 mM, the transformed *E. coli* cells were incubated at 28°C for 3 h. The bacteria were then spun down and to each of the pellets was added 1 mL of phosphate-buffered saline (PBS), containing 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.3 with 50 μL 0.2 M phenylmethylsulfonyl fluoride The pellets were then sonicated to lyse the bacteria. About 20 μg of lysate protein from each pellet was analyzed on an SDS gel and we found that use of 0.4 mM IPTG induced the highest titer of fusion protein. The crude lysate containing the fusion protein was then tested to see if it could convert [2,3-^{3}H]GA_9 and [17,17-^{2}H]GA_9 to labeled-GA_4. We used the bacterial lysate transformed with pGEX-2T vector alone as a control.

To make a large amount of recombinant AtGA3ox1 fusion protein, the transformed bacteria were inoculated into 100 mL of enriched medium (2% [w/v] tryptone; 1% [w/v] yeast extract; 50 mM KPi; 0.2% [w/v] glycerol; 0.5% [w/v] NaCl; pH 7.5) and incubated overnight at 37°C with shaking. Fresh medium (400 mL) was then used to dilute the bacteria and IPTG was added to a final concentration of 0.4 mM. After shaking at 28°C for 5 to 6 h, the bacteria were spun down at 4°C in 250-mL bottles at 8,000 rpm (approximately 5,000*g*) for 10 min. The pellet was suspended in 80 mL of PBS with 0.5 mM phenylmethylsulfonyl fluoride on ice. A French press was then used to lyse the bacteria in order to release the fusion protein and the lysate was centrifuged at 10,000 rpm (approximately 6,000*g*) for 10 min at 4°C to remove cell debris. The supernatant was passed through a preconditioned glutathione Sepharose 4B (2 mL) affinity column (Pharmacia, Piscataway, NJ). After washing the column with 20 mL of PBS, the flow was stopped and 10 mL of a freshly made reduced glutathione elution buffer (10 mM glutathione in 50 mM Tris, pH 7.5) was added. The affinity-column eluate was collected in 1-mL fractions and a Bio-Rad (Hercules, CA) protein assay was used to estimate protein titer for each fraction. The fusion protein eluted mainly in fractions 2 and 3, which were combined. Aliquots containing 100 μg of protein were taken and frozen in liquid N_2 for storage at −60°C.

Enzyme Assays

When the crude lysate was used, 90 μL were mixed with 5 μL of the 3-deoxy GA substrates and another 5 μL of the necessary cofactors (10 mM Fe^{2+}, 80 mM 2-oxoglutarate, 80 mM ascorbate, 2 mg bovine serum albumin [BSA] mL^{-1}, and 1 mg catalase mL^{-1}). When purified fusion protein was used, a thawed aliquot of the purified recombinant AtGA3ox1 fusion protein was added to 2 mL of Tris-HCl buffer (pH 7.5) containing 2 mg BSA mL^{-1}, 2 mM dithiothreitol, 2 mM nicotinamide adenine dinucleotide phosphate, reduced (NADPH), 5 mM 2-oxoglutarate, 5 mM of ascorbic acid, and 0.1 mM Fe^{2+}. The 3-deoxy GA substrates were first dissolved in methanol, then diluted to 10% methanol (v/v) with 50 mM Tris-HCl buffer (pH 7.5). Unless specified otherwise, all tests, including enzyme stability tests, were carried out at room temperature (20°C). The stability of the fusion protein was tested over 2, 5, 10, 16, 30, 60, and 120 min by taking an 80-μL aliquot of diluted protein from each incubation time and adding it to the 20 μL of 200 μM GA_{20} solution, followed by a 10-min reaction period. For kinetic studies, diluted protein was incubated at room temperature for 30 min before adding the 3-deoxy GA substrates to initiate the assay reaction. This was accomplished with and without the various ring D-modified GA_5 derivatives and ring D-modified GA_{20} derivatives, and with GA_5 (as putative competitive inhibitors of 3β-hydroxylation).

The assay reaction was stopped by adding 1 mL of cold 5% HOAc and mixing, yielding a pH of about 3. In order to measure the reaction product and/or precursor remaining, a known amount of internal standards was added and the mixture then partitioned 3× against 1 volume of H_2O-saturated EtOAc. An aliquot of the combined EtOAc fractions was dried under N_2, methylated with ethereal diazomethane, and trimethylsilyated by *N,O,*-bis(trimethylsilyl)trifluoroacetimide (BSTFA) with 1% trimethylchorosilane (TMCS) prior to analysis by GC-MS-SIM (Sheng et al., 1992). The GA substrate or product was then quantified by isotope dilution of the [$^2H_2$]GA (Fujioka et al., 1988). Identification was made by full-scan GC-MS.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

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