

# Characterization of the East Asian Variant of Aldehyde Dehydrogenase-2

## BIOACTIVATION OF NITROGLYCERIN AND EFFECTS OF Alda-1\*

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The East Asian variant of mitochondrial aldehyde dehydrogenase (ALDH2) exhibits significantly reduced dehydrogenase, esterase, and nitroglycerin (GTN) denitrating activities. The small molecule Alda-1 was reported to partly restore low acetaldehyde dehydrogenase activity of this variant. In the present study we compared the wild type enzyme (ALDH2\*1) with the Asian variant (ALDH2\*2) regarding GTN bioactivation and the effects of Alda-1. Alda-1 increased acetaldehyde oxidation by ALDH2\*1 and ALDH2\*2 approximately 1.5- and 6-fold, respectively, and stimulated the esterase activities of both enzymes to similar extent as the coenzyme NAD. The effect of NAD was biphasic with pronounced inhibition occurring at  $\geq 5$  mM. In the presence of 1 mM NAD, Alda-1 stimulated ALDH2\*2-catalyzed ester hydrolysis 73-fold, whereas the NAD-stimulated activity of ALDH2\*1 was inhibited because of 20-fold increased inhibitory potency of NAD in the presence of the drug. Although ALDH2\*2 exhibited 7-fold lower GTN denitrating activity and GTN affinity than ALDH2\*1, the rate of nitric oxide formation was only reduced 2-fold, and soluble guanylate cyclase (sGC) activation was more pronounced than with wild type ALDH2 at saturating GTN. Alda-1 caused slight inhibition of GTN denitration and did not increase GTN-induced sGC activation in the presence of either variant. The present results indicate that Alda-1 stimulates established ALDH2 activities by improving NAD binding but does not improve the GTN binding affinity of the Asian variant. In addition, our data revealed an unexpected discrepancy between GTN reductase activity and sGC activation, suggesting that GTN denitration and bioactivation may reflect independent pathways of ALDH2-catalyzed GTN biotransformation.

The antianginal drug nitroglycerin (glyceryl trinitrate (GTN))<sup>2</sup> causes vasodilation through NO-mediated activation of vascular sGC (1–3). The mechanism of GTN metabolism

resulting in NO formation is still elusive, but mitochondrial aldehyde dehydrogenase (ALDH2) has been identified as key enzyme catalyzing GTN bioconversion to 1,2-GDN and inorganic nitrite (4, 5). The established function of ALDH2 is the oxidative detoxification of ethanol-derived acetaldehyde and other aliphatic aldehydes. In addition, the enzyme catalyzes hydrolysis of various esters. GTN bioconversion has been proposed to be catalyzed through this esterase activity of ALDH2 (4). Interestingly, organic nitrates must contain at least three nitrate groups to be efficient substrates for ALDH2 (6). The most compelling evidence for an essential role of ALDH2 in GTN bioactivation was obtained with ALDH2 knock-out mice in which the vascular response to low doses of GTN was completely abolished, whereas the response to the NO donor sodium nitroprusside was preserved (7). The involvement of ALDH2 in GTN-induced vascular relaxation of rodent and human blood vessels has been confirmed in several subsequent *in vitro* and *in vivo* studies (for a recent review see Ref. 8). At low micromolar GTN concentrations the ALDH2-catalyzed reaction results in highly selective formation of 1,2-GDN, but this selectivity is lost at high substrate concentrations or in the presence of ALDH2 inhibitors (9, 10). The major nitrogen-containing product of GTN denitration is inorganic nitrite (4), but we found that ALDH2 additionally catalyzes formation of NO, detectable by a Clark-type NO electrode and as activation of purified sGC (11). Because inorganic nitrite could be excluded as intermediate, NO formation appears to result from direct enzymatic three-electron reduction of GTN by ALDH2. Detailed comparison of the initial reaction rates suggested that the three-electron reduction pathway normally accounts for ~10% of total GTN turnover. However, the fractional contribution of NO formation was increased to up to 50% upon mutation of the general base E268Q (12), pointing to an independent reaction that is not strictly coupled to (clearance-based) GTN denitration. Although direct formation of NO may explain how ALDH2-catalyzed GTN bioconversion is linked to vascular cGMP accumulation, this issue is still unsettled because several laboratories failed to detect NO formation in GTN-exposed blood vessels (13–15).

A dominant-negative ALDH2 polymorphism resulting in markedly reduced alcohol tolerance has been described (16–

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<sup>2</sup> The abbreviations used are: GTN, glyceryl trinitrate (nitroglycerin); sGC, soluble guanylate cyclase; DEA/NO, 2,2-diethyl-1-nitroso-oxyhydrazine; GDN,

glyceryl dinitrate; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; DTPA, diethylene triamine pentaacetic acid; SOD, superoxide dismutase; p-NPA, p-nitrophenyl acetate; NAD, nicotinamide adenine dinucleotide.

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18) that occurs with an incidence of 35–57% in different East Asian subpopulations (19). The East Asian variant of the enzyme (ALDH2\*2) has a lysine substituted for glutamate at position 487 (E487K), resulting in very low aldehyde dehydrogenase activity (20) caused by a local structural alteration that is transferred to both the active site and the NAD-binding domain (21, 22). In the presence of 0.5 mM NAD and 1  $\mu$ M GTN, the GTN reductase activity of ALDH2\*2 was reported to be 2 orders of magnitude lower than that of wild type ALDH2 (ALDH2\*1) (22). This observation is thought to explain the reduced hemodynamic response to GTN of humans carrying the ALDH2\*2 allele (23, 24).

Recently, high throughput screening led to the identification of ALDH2-activating compounds that are protective against cardiac ischemia (25). The most active compound, *N*-(1,3-benzodioxol-5-ylmethyl)-2,6-dichlorobenzamide (Alda-1 for aldehyde dehydrogenase activator-1), caused a moderate,  $\sim$ 2-fold stimulation of acetaldehyde oxidation by ALDH2\*1 but increased the residual aldehyde dehydrogenase activity of ALDH2\*2 11-fold. The mode of action of Alda-1 is still unknown.

In the present study we addressed two major issues. First we intended to compare the established activities of ALDH2\*2 with its ability to catalyze GTN denitration and/or bioactivation. The data revealed that the Asian variant catalyzes GTN bioactivation with similar maximal rates as the wild type enzyme, albeit with significantly lower GTN affinity. In light of these surprising results, it was of interest to see whether the bioactivation of pharmacologically relevant low concentrations of GTN could be improved by the ALDH2 activator Alda-1. In the second part of the study, we therefore thoroughly characterized the mode of action of Alda-1 with respect to established ALDH2 activities and GTN bioconversion.

### EXPERIMENTAL PROCEDURES

**Materials**—Bovine lung sGC was purified as previously described (26). [ $\alpha$ - $^{32}$ P]GTP (400 Ci/mmol) was obtained from Perkin Elmer Vertriebs GmbH (Vienna, Austria). [2- $^{14}$ C]GTN (55 mCi/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO) via Humos Diagnostika GmbH (Salzburg, Austria). Nitropohl<sup>®</sup> ampoules (G. Pohl-Boskamp GmbH & Co., Hohenlockstedt Germany) containing 4.4 mM GTN in 250 mM glucose were obtained from a local pharmacy; dilutions were made in 50 mM triethanolamine buffer (pH 7.4). DEA/NO was purchased from Alexis Corporation (Lausen, Switzerland) via Eubio (Vienna, Austria) and diluted in 10 mM NaOH. All other chemicals were from Sigma-Aldrich GmbH (Vienna, Austria).

**Synthesis of *N*-(1,3-Benzodioxol-5-ylmethyl)-2,6-dichlorobenzamide (Alda-1)**—2,6-Dichlorobenzoyl chloride (4.5 mmol) was added to a mixture of 3 mmol of piperonylamine and 4.5 mmol of triethylamine (deacidified over aluminum oxide) in 50 ml of CH<sub>2</sub>Cl<sub>2</sub> that had been distilled and dried over a 0.4-nm molecular sieve (Merck). The reaction batch was stirred overnight at room temperature in argon atmosphere, subsequently transferred to a separatory funnel, and shaken with 1 N NaOH. The aqueous phase was repeatedly extracted with CH<sub>2</sub>Cl<sub>2</sub>. The combined organic layers were washed with

H<sub>2</sub>O until the aqueous phase reacted neutral, dried over sodium sulfate, and filtered, and the solvent was evaporated *in vacuo*. The residue was recrystallized twice from ethanol and dried over phosphorus pentoxide at 50 °C *in vacuo*. The process yield was 94%, and the structure of the compound was verified by NMR spectroscopy (not shown). Stock solutions of Alda-1 (0.1 M in dimethyl sulfoxide) were diluted 1:100 with 50% dimethyl sulfoxide in H<sub>2</sub>O (v/v).

**ALDH2 Expression and Purification**—Human ALDH2\*1 was expressed in *Escherichia coli* BL21(DE3) using the pT7.7 expression system as previously described (27). To construct the human ALDH2\*2 variant, two complementary 30-mer primers (5'-CTGCAGGCcTACACTaAAGTGAA AACTGTC-3') were used for site-directed mutagenesis using the QuikChange<sup>®</sup> II Kit (Stratagene, La Jolla, CA), following the manufacturer's instructions. The primers contained the GCA to GCc silent point mutation to introduce a recognition site for the restriction enzyme StuI (underlined sequence), used to screen for positive clones, and the GAA to aAA point mutation to code for the Glu<sup>487</sup>-to-Lys substitution. ALDH2\*1 and ALDH2\*2 were purified by affinity and size exclusion chromatography as described previously (11).

**Determination of ALDH2 Dehydrogenase and Esterase Activities**—Dehydrogenase activity was measured as conversion of NAD (1 mM) to NADH in the presence of formaldehyde, acetaldehyde, or propionaldehyde (1 mM each) by monitoring the change in absorbance at 340 nm ( $\epsilon_{340} = 6.22 \text{ mM}^{-1} \times \text{cm}^{-1}$ ) at 25 °C (28). The reactions were performed in 50 mM sodium pyrophosphate buffer (pH 9.0) containing 10 mM MgCl<sub>2</sub> and 10  $\mu$ M Alda-1 or 0.5% (v/v) DMSO as a vehicle control. Enzyme activities were calculated as the mean values  $\pm$  S.E. from three independent determinations.

Esterase activity was measured by monitoring formation of *p*-nitrophenolate from 0.1 mM *p*-NPA ( $\epsilon_{400} = 16 \text{ mM}^{-1} \times \text{cm}^{-1}$ ) in 50 mM sodium pyrophosphate buffer (pH 7.5) containing 10 mM MgCl<sub>2</sub>, 10  $\mu$ M Alda-1, or 0.5% DMSO and varying concentrations (0–20 mM) of NAD (29). Enzyme activities were calculated as the mean values  $\pm$  S.E. from three independent determinations.

**Kinetics of ALDH2 Inactivation by GTN**—GTN-induced inactivation of the two ALDH2 variants was assayed in the absence and presence of 10  $\mu$ M Alda-1 as described previously (30). Dehydrogenase activity was measured by monitoring the formation of NADH as increase in light absorbance at 340 nm in 50 mM sodium pyrophosphate buffer (pH 7.5) containing 0.2 mM acetaldehyde, 5 mM NAD, 10 mM MgCl<sub>2</sub>, and 10  $\mu$ M Alda-1 or 0.5% (v/v) DMSO as vehicle control. After 2 min of equilibration, the reactions were started by the addition of ALDH2\*1 or ALDH2\*2 (37 and 111  $\mu$ g/ml final concentrations, respectively) and monitored for  $\sim$ 4 min to obtain initial reaction rates ( $v_0$ ), followed by the addition of GTN (0.2 mM final concentration) for determination of inactivation rate constants ( $k_{\text{inact}}$ ) by fitting the data to a single-exponential curve. After complete enzyme inactivation, 1 mM DTT was added for determination of restored activities ( $v_{\text{restored}}$ ).

**Determination of GTN Denitration by Radio Thin Layer Chromatography**—GTN denitration yielding 1,2- and 1,3-GDN was measured as described previously (9). Purified

ALDH2\*1 or ALDH2\*2 (4  $\mu\text{g}$  each) was incubated with  $^{14}\text{C}$ -radiolabeled GTN (2  $\mu\text{M}$ ,  $\sim 50,000$  dpm) at 37 °C for 10 min in a final volume of 200  $\mu\text{l}$  of 50 mM phosphate buffer (pH 7.4) containing 3 mM  $\text{MgCl}_2$ , 2 mM DTT, 1 mM EDTA, 1 mM EGTA, and unlabeled GTN as required to obtain the indicated final concentrations. The amount of added radioactivity was increased 2-fold for determination of reaction rates at GTN concentrations  $\geq 30$   $\mu\text{M}$  and 3-fold at GTN concentrations of  $\geq 200$   $\mu\text{M}$ . NAD (1 mM) and Alda-1 (10  $\mu\text{M}$ ) or DMSO (0.5% v/v) were present as indicated in the text and figure legends. The reaction products were extracted twice with 1 ml of diethyl ether, separated by thin layer chromatography, and quantified by liquid scintillation counting. The blank values were determined in the absence of protein under identical conditions and subtracted.

Apparent  $K_m$  values were determined by fitting the plots of the activity as a function of the GTN concentration to the Michaelis-Menten equation, disregarding the activities measured in the presence of 1 mM GTN. The values indicated under "Results" are the mean values  $\pm$  S.E. of three determinations.

**Determination of sGC Activity**—Purified bovine lung sGC (50 ng) was incubated at 37 °C in a final volume of 0.1 ml with the indicated concentrations of GTN in the presence of ALDH2\*1 or ALDH2\*2. Maximal sGC activation was determined in the presence of 10  $\mu\text{M}$  DEA/NO. Assay mixtures contained 50 mM triethanolamine (pH 7.4), 3 mM  $\text{MgCl}_2$ , 0.5 mM [ $\alpha$ - $^{32}\text{P}$ ]GTP ( $\sim 250,000$  cpm), 1 mM cGMP, 2 mM DTT, and 0.1 mM DTPA. The enzymes and GTN were present as specified in the text and figure legends. When indicated, NAD (1 mM) and Alda-1 (10  $\mu\text{M}$ ) or DMSO (0.5% v/v) were additionally present. The reactions were terminated by the addition of 0.45 ml of zinc acetate (120 mM) and 0.45 ml of sodium bicarbonate (120 mM), followed by the separation and quantification of  $^{32}\text{P}$ -cGMP as described previously (31). Blank values determined in the absence of sGC were subtracted.

**Determination of GTN-derived NO in the Presence of ALDH2**—NO formation was measured with a Clark-type electrode (World Precision Instruments, Berlin, Germany) calibrated daily with acidified nitrite as described previously (32) in a total volume of 0.5 ml containing 50 mM triethanolamine (pH 7.4), 1000 units/ml SOD, 2 mM DTT, 3 mM  $\text{MgCl}_2$ , 0.1 mM DTPA, and 125  $\mu\text{g}$  of purified ALDH2\*1 or ALDH2\*2. After equilibrating the solution at 37 °C for 10 min, GTN was added to give a final concentration of 1 or 100  $\mu\text{M}$ .

**Correlation between ALDH Inactivation and the Time Course of Apparent NO Decay**—For a NO-generating enzyme operating under  $V_{\text{max}}$  conditions, one would expect the NO concentration in the NO electrode experiments to reach a steady state that is determined by Equation 1,

$$v_{\text{NO}} = k_{\text{cat}} \cdot E - k_{\text{auto}} \cdot [\text{NO}]^2 \cdot [\text{O}_2] - k_{\text{diff}} \cdot [\text{NO}] = 0 \quad (\text{Eq. 1})$$

where  $v_{\text{NO}}$  is the net rate of NO formation,  $k_{\text{cat}}$  is the maximal turnover rate of the enzyme,  $E$  is the enzyme (ALDH) concentration,  $k_{\text{auto}}$  is the rate constant for autooxidation of NO, and  $k_{\text{diff}}$  is the apparent rate constant for diffusion of NO out of the solution (interphase mass transfer), including presumably

minor NO consumption at the electrode. If the enzyme is inactivated during measurement, the same equation still applies, except that the total enzyme concentration ( $E$ ) has to be replaced by the active enzyme concentration ( $E_{\text{act}}$ ). With  $E_{\text{act}} = k_{\text{cat}} \cdot E \cdot \exp(-k_{\text{inact}} \cdot t)$  and  $k_{\text{inact}}$  representing the rate constant for inactivation of ALDH, this yields Equation 2.

$$k_{\text{cat}} \cdot E \cdot \exp(-k_{\text{inact}} \cdot t) = k_{\text{auto}} \cdot [\text{NO}]^2 \cdot [\text{O}_2] + k_{\text{diff}} \cdot [\text{NO}] \quad (\text{Eq. 2})$$

On the right-hand side of Equation 2 autooxidation will predominate at high NO concentrations, whereas the diffusional term will prevail at low concentrations. For the first case one can derive that the NO signal will decay over time according to Equation 3.

$$[\text{NO}] = (k_{\text{cat}} \cdot E_0 / k_{\text{auto}} \cdot [\text{O}_2])^{1/2} \times \exp(-(k_{\text{inact}}/2) \cdot t) = C_1 \cdot \exp(-(k_{\text{inact}}/2) \cdot t) \quad (\text{Eq. 3})$$

Consequently, the NO signal is expected to disappear with an apparent rate constant of  $k_{\text{inact}}/2$ . For lower NO concentrations, a similar equation (Equation 4) can be derived, which only differs from Equation 3 by the absence of the factor  $1/2$ .

$$[\text{NO}] = (k_{\text{cat}} \cdot E_0 / k_{\text{diff}}) \times \exp(-k_{\text{inact}} \cdot t) = C_2 \cdot \exp(-k_{\text{inact}} \cdot t) \quad (\text{Eq. 4})$$

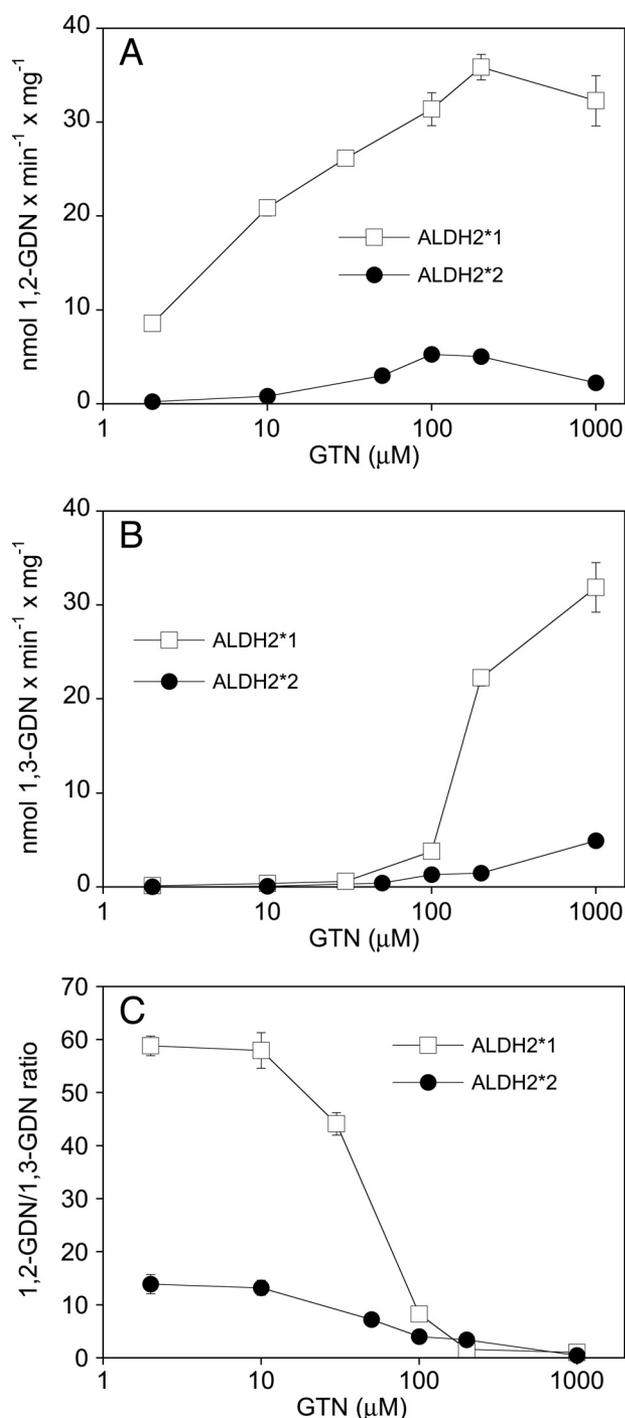
Both Equations 3 and 4 predict that ALDH2 inactivation should result in first order decay of the NO signal with the observed rate constant corresponding to  $(0.5-1.0) \times k_{\text{inact}}$ .

## RESULTS

**GTN Denitration**—Fig. 1A shows that ALDH2\*2 catalyzed GTN denitration with  $\sim 7$ -fold lower maximal rates than ALDH2\*1 ( $5.0 \pm 0.10$  versus  $36 \pm 1.4$  nmol of 1,2-GDN  $\times$  min $^{-1} \times$  mg $^{-1}$  in the presence of 0.2 mM GTN). In addition, the apparent GTN affinity of ALDH2\*2 was  $\sim 7$ -fold lower (apparent  $K_m$  values of  $54 \pm 6.0$  and  $7.6 \pm 1.4$   $\mu\text{M}$  for ALDH2\*2 and ALDH2\*1, respectively), resulting in pronounced, 35-fold lower rates of GTN reduction at pharmacologically relevant low concentrations of the nitrate ( $0.24 \pm 0.02$  versus  $8.6 \pm 0.19$  nmol 1,2-GDN  $\times$  min $^{-1} \times$  mg $^{-1}$  for ALDH2\*2 and ALDH2\*1 in the presence of 2  $\mu\text{M}$  GTN). As shown in Fig. 1B, neither of the two forms of the enzyme generated significant amounts of 1,3-GDN at  $< 0.1$  mM GTN. At high GTN concentrations, ALDH2\*2 exhibited much lower rates of 1,3-GDN formation than the wild type enzyme ( $5.0 \pm 0.01$  versus  $32 \pm 8.2$  nmol  $\times$  min $^{-1} \times$  mg $^{-1}$  at 1 mM GTN). The data were used to calculate the 1,2-/1,3-GDN ratios (Fig. 1C), indicating that ALDH2\*1 catalyzed denitration of 2  $\mu\text{M}$  GTN with  $> 98\%$  selectivity for the 1,2-isomer. With increasing concentrations of GTN, the 1,2-/1,3-GDN ratio gradually decreased ( $1.0 \pm 0.08$  at 1 mM GTN). With ALDH2\*2, the 1,2-/1,3-GDN ratios were  $14 \pm 0.01$  and  $0.45 \pm 0.10$  at 2  $\mu\text{M}$  and 1 mM GTN, respectively.

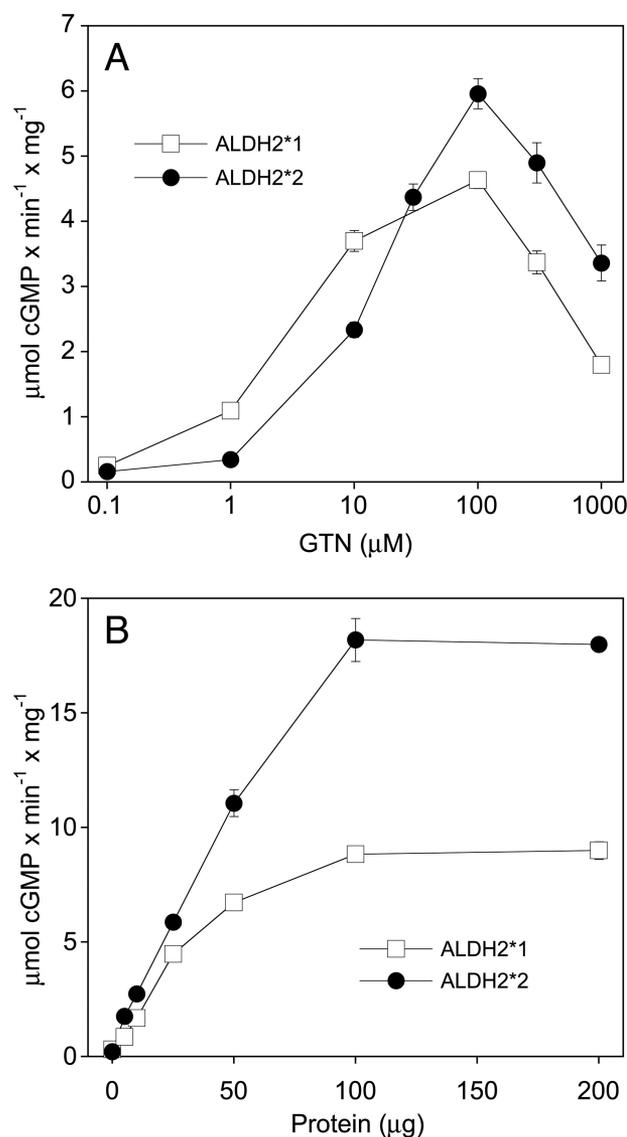
**GTN Bioactivation**—Bioactivation of GTN was assayed by coincubation of ALDH2 with purified sGC and determination of GTN-induced cGMP formation. As shown in Fig. 2A, GTN caused biphasic activation of sGC in the presence of both

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**FIGURE 1. Effects of GTN concentration on the formation of 1,2-GDN (A) and 1,3-GDN (B) and 1,2-/1,3-GDN ratios (C).** ALDH2\*1 and ALDH2\*2 (4 μg each) were incubated at 37 °C for 10 min in the presence of 3 mM MgCl<sub>2</sub>, 2 mM DTT, 1 mM NAD, 1 mM EDTA, 1 mM EGTA and the indicated concentration of [<sup>14</sup>C]GTN. Reaction products were extracted and quantified by radio thin layer chromatography as described in "Experimental Procedures". Data are mean values ± S.E. of three independent experiments.

ALDH2 variants with apparent EC<sub>50</sub> values of 3.4 ± 0.20 and 15 ± 1.5 μM for ALDH2\*1 and ALDH2\*2, respectively. Although the ~5-fold lower apparent GTN affinity of the East Asian variant agrees well with the denitration data shown in Fig. 1A, the even slightly increased maximal effect on cGMP caused by ALDH2\*2 (6.0 ± 0.23 versus 4.6 ± 0.12 μmol

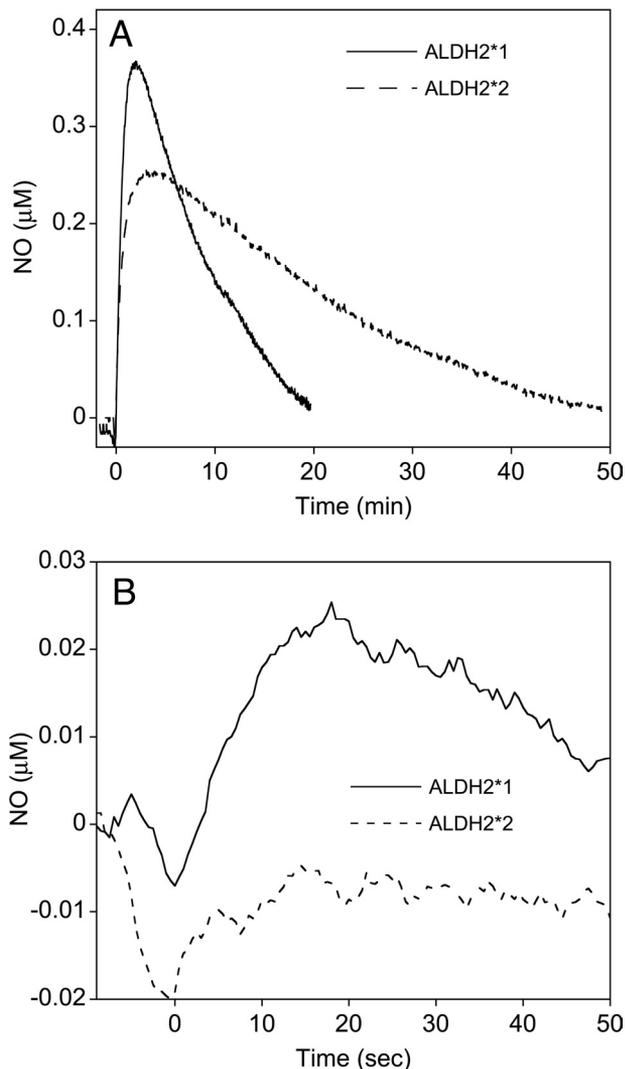


**FIGURE 2. Dependence of sGC activation on GTN (A) and ALDH2 (B) concentration.** Purified sGC (50 ng) was incubated at 37 °C for 10 min in the presence of 0.5 mM [α-<sup>32</sup>P]GTP (~250 000 cpm), 3 mM MgCl<sub>2</sub>, 1 mM cGMP, 2 mM DTT and 0.1 mM DTPA. A: Activation of sGC by increasing concentrations of GTN in the presence of ALDH2\*1 or ALDH2\*2 (25 μg each). B: Activation of sGC by 0.1 mM GTN in the presence of the indicated amounts of ALDH2\*1 or ALDH2\*2. [<sup>32</sup>P]cGMP was isolated and quantified as described in "Experimental Procedures". Data are mean values ± S.E. of three independent experiments.

cGMP × min<sup>-1</sup> × mg<sup>-1</sup> at 0.1 mM GTN) was unexpected considering the lower GTN reductase activity of the Asian variant. The apparent discrepancy between GTN denitration and sGC activation became even more evident at higher ALDH2 concentrations. Formation of cGMP increased with both enzymes until a limit was reached at ~100 μg of ALDH2 (Fig. 2B). However, whereas ALDH2\*2 caused maximal sGC activation (18 ± 0.94 μmol cGMP × min<sup>-1</sup> × mg<sup>-1</sup>) as determined with the NO donor DEA/NO (20 ± 0.06 μmol × min<sup>-1</sup> × mg<sup>-1</sup>), the maximal effect of ALDH2\*1 was 2-fold lower (9.0 ± 0.38 μmol × min<sup>-1</sup> × mg<sup>-1</sup>).

Based on recent results indicating that ALDH2-catalyzed GTN bioconversion is associated with superoxide formation (12), we measured sGC activation by 0.1 mM GTN in the pres-

ence of 100  $\mu\text{g}$  of the two ALDH2 variants and 1,000 units/ml SOD. Under these conditions the rates of cGMP formation were  $24 \pm 1.2$  and  $24 \pm 0.93 \mu\text{mol} \times \text{min}^{-1} \times \text{mg}^{-1}$  in the presence of ALDH2\*1 and ALDH2\*2, respectively (mean values  $\pm$  S.E.;  $n = 3$ ). Thus, in the presence of SOD both variants led to sGC activation by GTN that was even slightly more pronounced than the maximal effect of the NO donor DEA/NO ( $21 \pm 0.26 \mu\text{mol cGMP} \times \text{min}^{-1} \times \text{mg}^{-1}$ ).



**FIGURE 3. Formation of GTN-derived NO by ALDH2.** Purified ALDH2\*1 or ALDH2\*2 (25  $\mu\text{g}/0.1$  ml each) was incubated at 37  $^{\circ}\text{C}$  in a total volume of 0.5 ml containing 50 mM TEA (pH 7.4), 1000 units/ml SOD, 2 mM DTT, 3 mM  $\text{MgCl}_2$ , 0.1 mM DTPA. After equilibrating the solution at 37  $^{\circ}\text{C}$  for 10 min, GTN was added to give final concentrations of 100  $\mu\text{M}$  (A) or 1  $\mu\text{M}$  (B). NO formation was monitored with a Clark-type electrode as described in "Experimental Procedures". The traces shown are the average of three recordings.

**TABLE 1**

**Effects of Alda-1 on oxidation of different aldehyde substrates by ALDH2\*1 and ALDH2\*2**

Dehydrogenase activity was determined as described under "Experimental Procedures" as conversion of NAD (1 mM) to NADH in the presence of formaldehyde, acetaldehyde, and propionaldehyde (1 mM each) at pH 9.0. The data are the mean values  $\pm$  S.E. of specific activities ( $\text{nmol of NADH} \times \text{min}^{-1} \times \text{mg}^{-1}$ ) and relative activities (Alda-1 vs. control) determined in three independent experiments.

Substrate	ALDH2*1 (E487)			ALDH2*2 (K487)		
	Control	With Alda-1	Relative activity	Control	With Alda-1	Relative activity
Formaldehyde	$3000 \pm 80$	$290 \pm 6.9$	$0.097 \pm 0.0022$	$3.6 \pm 0.043$	$8.4 \pm 0.06$	$2.3 \pm 0.021$
Acetaldehyde	$1500 \pm 67$	$2200 \pm 11$	$1.5 \pm 0.015$	$13 \pm 0.87$	$79 \pm 1.9$	$6.0 \pm 0.11$
Propionaldehyde	$1000 \pm 25$	$1700 \pm 96$	$1.70 \pm 0.10$	$21 \pm 1.1$	$75 \pm 1.0$	$3.6 \pm 0.055$

The two ALDH2 variants exhibited significantly different kinetics of GTN-derived NO formation, determined electrochemically with a Clark-type electrode in the presence of SOD. As shown in Fig. 3A, both forms of the enzyme evoked pronounced NO signals in the presence of saturating GTN concentrations (0.1 mM) with apparent initial rates of  $1.7 \pm 0.06$  and  $0.83 \pm 0.04 \text{ nmol} \times \text{min}^{-1} \times \text{mg}^{-1}$ , respectively. The maximal NO concentration measured with the wild type enzyme ( $0.37 \pm 0.05 \mu\text{M}$ ) was reached  $120 \pm 3.0$  s after injection of GTN and rapidly decayed, whereas the maximum for ALDH2\*2 ( $0.26 \pm 0.03 \mu\text{M}$ ) was obtained  $290 \pm 8.2$  s after injection, and decay was much slower. The signals obtained with both enzymes were immediately reduced to base line upon addition of 10  $\mu\text{M}$  of the NO scavenger oxyhemoglobin (data not shown). The decay of the signals could be fitted to single exponentials with apparent rate constants ( $k_{\text{obs}}$ ) of  $19 \pm 0.12 \times 10^{-4} \times \text{s}^{-1}$  and  $5.6 \pm 0.08 \times 10^{-4} \times \text{s}^{-1}$  for ALDH2\*1 and ALDH2\*2, respectively. Because NO inactivation by superoxide was prevented by including a large amount of SOD (1,000 units/ml), these results suggest that ALDH2\*2 inactivates more slowly during turnover and forms larger amounts of NO from saturating GTN than the wild type enzyme. A different picture was obtained, however, when NO formation was measured at a pharmacologically more relevant low concentration of GTN (1  $\mu\text{M}$ ). As shown in Fig. 3B, ALDH2 generated a NO signal with a peak corresponding to a concentration of  $\sim 20$  nM under these conditions, whereas formation of GTN-derived NO by ALDH2\*2 was not detectable with the NO electrode. Thus, the low apparent GTN affinity renders the Asian mutant significantly less efficient than the wild type enzyme at pharmacologically relevant low GTN concentrations.

**Effects of Alda-1 on Dehydrogenase Activities**—Table 1 shows the rates of oxidation of different aldehyde substrates by the two ALDH2 variants in the absence and presence of 10  $\mu\text{M}$  Alda-1. As described previously (33–35), the activity of ALDH2\*1 decreased with the size of the substrate (from  $3000 \pm 80$  to  $1000 \pm 25 \text{ nmol} \times \text{min}^{-1} \times \text{mg}^{-1}$  with formaldehyde and propionaldehyde, respectively). In contrast, the activity of ALDH2\*2 increased from  $3.6 \pm 0.04 \text{ nmol} \times \text{min}^{-1} \times \text{mg}^{-1}$  with formaldehyde to  $21 \pm 1.1 \text{ nmol} \times \text{min}^{-1} \times \text{mg}^{-1}$  with propionaldehyde. In line with published observations (25), 10  $\mu\text{M}$  Alda-1 stimulated oxidation of 1 mM acetaldehyde by ALDH2\*1 and ALDH2\*2 1.5- and 6-fold, respectively. Note that even in the presence of the activator, the specific activity of the East Asian variant was still 20-fold lower than that of the wild type enzyme. At 10 mM acetaldehyde, stimulation of ALDH2\*1 was similar (1.8-fold), but stimulation of the Asian variant was significantly more pronounced (15-fold; data not shown). Qual-

TABLE 2

## Effects of Alda-1 on GTN-induced inactivation of ALDH2\*1 and ALDH2\*2

GTN-induced inactivation of the two ALDH2 variants was assayed with 10  $\mu\text{M}$  Alda-1 or 0.5% (v/v) DMSO as vehicle control in the presence of 0.2 mM acetaldehyde, 5 mM NAD, and 10 mM  $\text{MgCl}_2$ . After 2 min of equilibration, the reactions were started by the addition of ALDH2\*1 or ALDH2\*2 (37 and 111  $\mu\text{g}/\text{ml}$  final concentration, respectively) and monitored for approximately 4 min to obtain initial reaction rates ( $v_0$ ), followed by the addition of GTN (200  $\mu\text{M}$  final concentration) for determination of inactivation rate constants ( $k_{\text{inact}}$ ) by fitting to a single exponential curve. After complete enzyme inactivation, 1 mM DTT was added for determination of restored activities ( $v_{\text{restored}}$ ). The data are the mean values  $\pm$  S.E. of three independent experiments.

Condition	$v_0$ $\text{nmol} \times \text{min}^{-1} \times \text{mg}^{-1}$	$k_{\text{inact}}$ $\text{s}^{-1}$	$v_{\text{restored}}$ $\text{nmol} \times \text{min}^{-1} \times \text{mg}^{-1}$
<b>ALDH2*1</b>			
Control	600 $\pm$ 20	8.2 $\pm$ 0.44 $\times 10^{-3}$	140 $\pm$ 4.9
Alda-1	630 $\pm$ 1.5	11 $\pm$ 0.67 $\times 10^{-3}$	150 $\pm$ 13
<b>ALDH2*2</b>			
Control	9.6 $\pm$ 0.056 <sup>a</sup>	2.5 $\pm$ 0.24 $\times 10^{-3}$ <sup>a</sup>	2.2 $\pm$ 0.25 <sup>a</sup>
Alda-1	40 $\pm$ 2.7 <sup>a,b</sup>	8.0 $\pm$ 0.47 $\times 10^{-3}$ <sup>c,d</sup>	6.8 $\pm$ 1.5 <sup>a</sup>

<sup>a</sup>  $p < 0.005$  vs. ALDH2\*1.

<sup>b</sup>  $p < 0.005$  vs. control.

<sup>c</sup>  $p < 0.001$  vs. control.

<sup>d</sup>  $p < 0.01$  vs. ALDH2\*1 (analysis of variance with a Scheffe test).

itatively similar results were obtained for oxidation of 1 mM propionaldehyde (1.7- and 3.6-fold stimulation of ALDH2\*1 and ALDH2\*2, respectively). However, oxidation of formaldehyde was differently affected by Alda-1; the drug inhibited the apparent activity of ALDH2\*1 by  $\sim 90\%$  but stimulated the ALDH2\*2-catalyzed reaction 2.4-fold.

**Effects of Alda-1 on GTN-triggered Inactivation of Dehydrogenase Activity**—Exposure of ALDH2 to GTN results in mechanism-based oxidative inactivation of the enzyme that is partially reversed by the reducing agent DTT (30). Table 2 shows initial acetaldehyde dehydrogenase activities ( $v_0$ ), the rate constants for enzyme inactivation induced by 0.2 mM GTN ( $k_{\text{inact}}$ ), and dehydrogenase activities restored by DTT, measured with the two ALDH2 variants in the absence and presence of 10  $\mu\text{M}$  Alda-1. Interestingly, GTN-induced inactivation of ALDH2\*2 was  $\sim 3$ -fold slower than inactivation of the wild type enzyme, and Alda-1 increased the inactivation rate constant to a value approaching that of wild type (from 2.5  $\pm$  0.24 to 8.0  $\pm$  0.47  $\times 10^{-3}$   $\times \text{s}^{-1}$ ). Neither the E487K substitution nor the presence of Alda-1 had significant effects on the recovery of the GTN-inactivated enzymes by DTT ( $\sim 20\%$  of  $v_0$ ).

**Effects of Alda-1 on Esterase Activities**—Esterase activities were measured photometrically as *p*-NPA hydrolysis in the absence and presence of 1 mM NAD, 10  $\mu\text{M}$  Alda-1, or a combination of both. As shown in Fig. 4A, the activities of ALDH2\*1 and ALDH2\*2 (190  $\pm$  5.5 and 0.21  $\pm$  0.01  $\text{nmol} \times \text{min}^{-1} \times \text{mg}^{-1}$ , respectively) were increased by NAD and Alda-1 to similar extent (6- and 9-fold, respectively). In the combined presence of NAD and Alda-1, however, the effect of either compound on wild type ALDH2 was almost abolished (1.7-fold stimulation), whereas the esterase activity of ALDH2\*2 was stimulated 73-fold (note that the esterase activity of ALDH2\*2 was still 20-fold lower than that of ALDH2\*1 under these conditions). The peculiar discrepancy between the effects of the Alda-1/NAD combination on the two ALDH2 variants is apparently due to a biphasic effect of NAD on the esterase activity of ALDH2. As shown in Fig. 4B, the esterase activities of ALDH2\*1 and ALDH2\*2 were maximally stimulated by 1 and 5

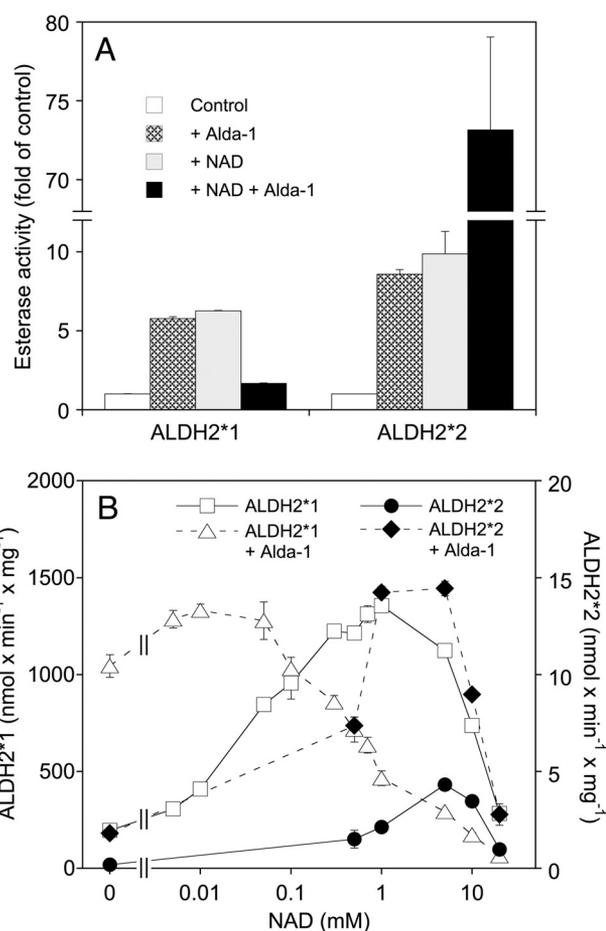
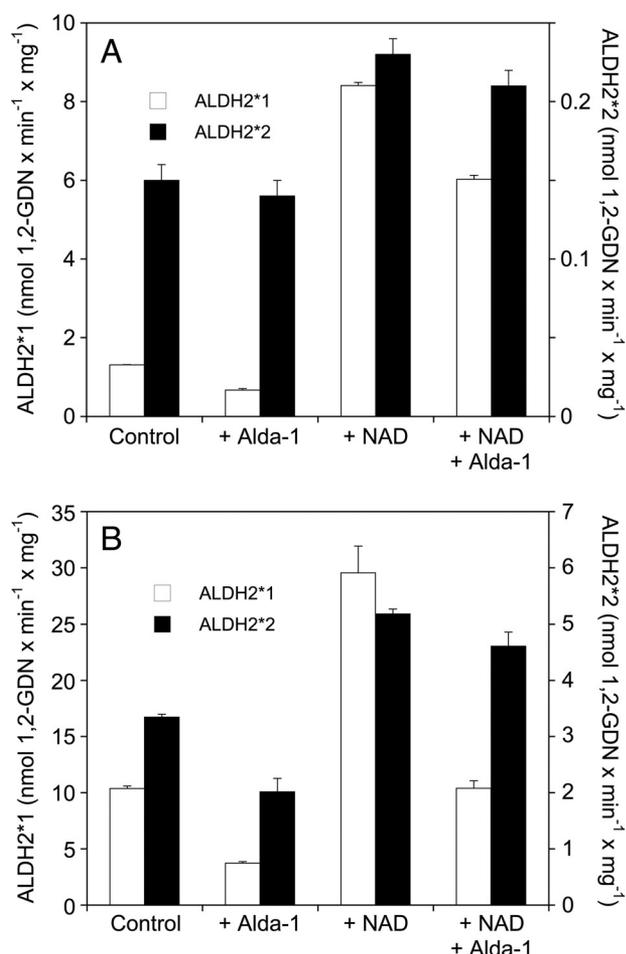


FIGURE 4. Effect of Alda-1 and NAD on the esterase activities of ALDH2\*1 and ALDH2\*2. Esterase activity was measured photometrically with 0.1 mM *p*-NPA as described in "Experimental Procedures" in the absence or presence of 1 mM (A) or increasing concentrations (B) NAD and 10  $\mu\text{M}$  Alda-1 or 0.5% DMSO (vehicle control). Activities were calculated as fold increase over controls (mean values  $\pm$  S.E.) obtained in three independent determinations. Note that the control value of ALDH2\*2 is only 0.11% of the ALDH2\*1 control.

mM NAD, respectively, whereas higher concentrations of the coenzyme led to marked inhibition of both ALDH2 variants. Alda-1 stimulated the esterase activities of both enzymes in the absence of NAD and potentiated the effect of the coenzyme, in particular its effect on ALDH2\*2. Intriguingly, Alda-1 additionally caused a pronounced  $\sim 20$ -fold left shift of NAD-induced inhibition of wild type ALDH2, which was half-maximal at  $\sim 10$  and 0.5 mM in the absence and presence of Alda-1.

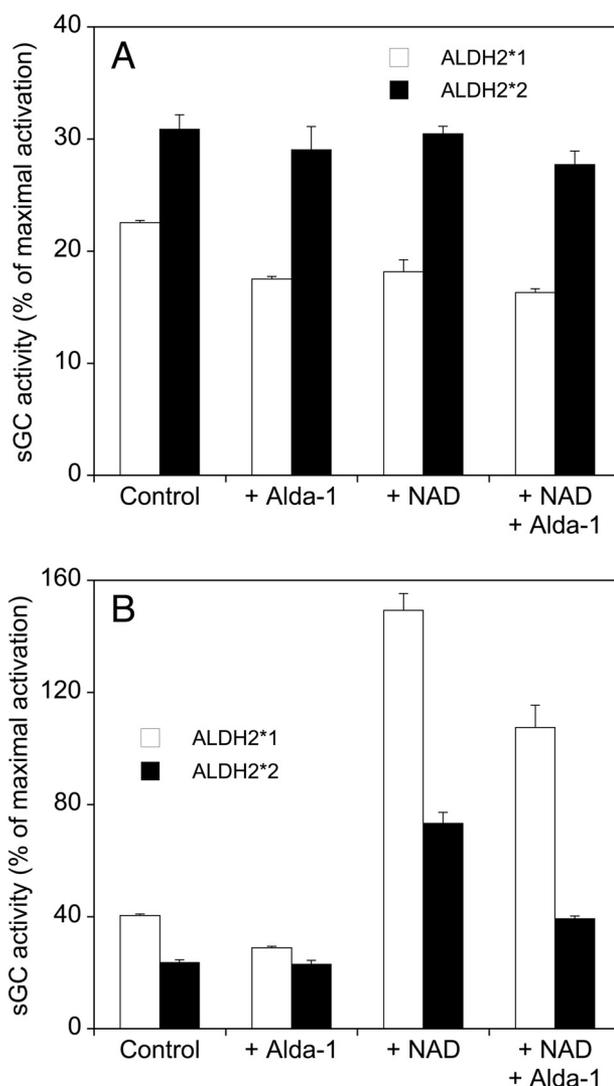
**Effects of Alda-1 on GTN Denitration and sGC Activation**—It was of interest to see whether Alda-1 is able to boost GTN denitration and bioactivation. Fig. 5 shows denitration of 2  $\mu\text{M}$  (panel A) and 100  $\mu\text{M}$  (panel B) GTN in the absence and presence of NAD (1 mM) or Alda-1 (10  $\mu\text{M}$ ) or a combination of both compounds. Maximal GTN reductase activity (30  $\pm$  2.4  $\text{nmol} \times \text{min}^{-1} \times \text{mg}^{-1}$  in the presence of 100  $\mu\text{M}$  GTN) and the degree of enzyme stimulation by NAD (6.5- and 2.8-fold in the presence of 2 and 100  $\mu\text{M}$  GTN, respectively) agree well with published data (4, 9). At a concentration of 1 mM, NAD had a less pronounced effect on GTN denitration by ALDH2\*2 ( $\sim 1.5$ -fold at both low and high GTN), but at 20 mM the nucleotide caused an approximately 3-fold increase in reaction rates (data not shown), reflecting the lower NAD affinity of the East Asian



**FIGURE 5. Effect of Alda-1 and NAD on GTN denitration.** ALDH2\*1 and ALDH2\*2 (4  $\mu\text{g}$  each) were incubated as described in the legend to Fig. 1 with 2  $\mu\text{M}$  (A) or 100  $\mu\text{M}$  (B) GTN, in the absence or presence of 1 mM NAD and 10  $\mu\text{M}$  Alda-1 or 0.5% DMSO (vehicle control). Reaction products were extracted and quantified by radio thin layer chromatography as described under "Experimental Procedures". Data represent mean values for 1,2-GDN formation  $\pm$  S.E. of three independent experiments. Note the different scales for the activities of ALDH2\*1 and ALDH2\*2.

variant (20, 22). In contrast to its effects on acetaldehyde oxidation and ester hydrolysis, Alda-1 did not increase the rates of GTN denitration by either ALDH2 variant but caused inhibition (Fig. 5B).

As shown in Fig. 6A, the rates of cGMP formation triggered by saturating concentrations of GTN (0.1 mM) in the absence of SOD were hardly changed by NAD, Alda-1, or a combination of both compounds. To obtain information on the effects of the nicotinamide and, in particular, Alda-1 on bioactivation of a pharmacologically relevant GTN concentration, the same set of experiments was performed with 1  $\mu\text{M}$  GTN in the presence of SOD (to exclude effects of superoxide on NO bioavailability). Upon short term incubation (1 min), ALDH2\*1 caused approximately half-maximal sGC activation in the presence of 1  $\mu\text{M}$  GTN and 1 mM NAD (Fig. 6B). Under these conditions, the effect of ALDH2/GTN even exceeded that of 10  $\mu\text{M}$  DEA/NO. The observation that NAD increases reaction rates only at low GTN confirms our previous results on the GTN-competitive effect of NAD (9, 12). As expected from the lower GTN affinity of the mutant, the



**FIGURE 6. Effect of Alda-1 and NAD on sGC activation.** Purified sGC was incubated with 25  $\mu\text{g}$  of ALDH2\*1 or ALDH2\*2 as described in the legend to Fig. 2A with 100  $\mu\text{M}$  GTN for 10 min (A) or 1  $\mu\text{M}$  GTN for 1 min (B) in the absence or presence of 1 mM NAD and 10  $\mu\text{M}$  Alda-1 or 0.5% DMSO (vehicle control). The experiments shown in panel B were performed in the presence of 1,000 units/ml SOD to exclude superoxide-mediated inactivation of NO.  $^{32}\text{P}$ -cGMP was isolated and quantified as described under "Experimental Procedures". Data are mean values  $\pm$  S.E. of three independent experiments.

rates of cGMP formation were much lower in the presence of ALDH2\*2. Alda-1 did not increase the efficiency of the Asian variant but slightly inhibited sGC activation by both enzymes in the absence and presence of NAD.

## DISCUSSION

**Effects of the E487K Substitution on ALDH2 Activities**—Our activity data agree well with detailed enzyme kinetic studies (20, 22) showing that the E487K substitution in human ALDH2 results in strongly reduced  $V_{\text{max}}$  values for all known activities of the enzyme (aldehyde oxidation, ester hydrolysis, GTN denitration) and more than 100-fold decreased affinity for NAD binding ( $K_m$  of 7.4 mM at pH 7.4). Thus, limited cofactor availability may be a major cause for the low dehydrogenase and esterase activities of ALDH2\*2 measured under our experimental conditions. In addition, the E487K substitution was reported

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to progressively lower  $V_{\max}$  of aldehyde oxidation with decreasing substrate size (36), presumably explaining our observation that residual activity of ALDH2\*2 was highest with propionaldehyde (2.1% of wild type), followed by oxidation of acetaldehyde and formaldehyde (0.83 and 0.12% of wild type, respectively). However, because formaldehyde exhibits low affinity even for wild type ALDH2 ( $K_m \sim 0.6$  mM) (33, 34), decreased substrate affinity may significantly contribute to the extremely low activity of ALDH2\*2 with formaldehyde.

The effect of the E487K substitution on GTN reductase activity decreased with increasing GTN concentration (3 and 15% of wild type with 2 and 100  $\mu$ M GTN, respectively; see Fig. 1A). This may reflect low affinity of ALDH2\*2 for GTN rather than for NAD, because GTN reductase activity is only moderately increased by the coenzyme (Fig. 5). At low GTN, the ALDH2-catalyzed reaction results in highly selective formation of 1,2-GDN, but this selectivity is largely lost at increasing GTN concentrations or in the presence of enzyme inhibitors (9). Similarly, inactivation of ALDH2 in nitrate-tolerant blood vessels results in the loss of 1,2-GDN selectivity (10, 37, 38). Formation of 1,3-GDN appears to occur through a nonspecific side reaction of the enzyme, which does not involve binding of GTN to the catalytic site because it is insensitive to substrate-competitive inhibitors. Interestingly, ALDH2\*2 also formed less 1,3-GDN than the wild type enzyme, but the 1,2-/1,3-GDN ratio was markedly decreased (Fig. 1C), suggesting a loss of specificity upon the E487K substitution.

**Effects of Alda-1 on Aldehyde Oxidation**—In line with published observations (25), we found that Alda-1 only moderately activated wild type ALDH2 (1.5-fold increase in acetaldehyde oxidation), whereas the residual activity of the East Asian variant was increased 6-fold. Similar results were obtained with propionaldehyde, but Alda-1 inhibited formaldehyde oxidation by ALDH2\*1 by  $\sim 90\%$  and caused only 2.3-fold stimulation of the ALDH2\*2-catalyzed reaction (Table 1). Interference of Alda-1 with substrate binding may explain the aberrant results with formaldehyde, because the relatively high affinities of the longer chain aldehydes may ensure saturation even without Alda-1. In agreement with this hypothesis, hardly any inhibition of ALDH2\*1 by Alda-1 was observed when the formaldehyde concentration was increased from 1 to 15 mM (90.2% of control; data not shown). Under the assumption that the effect of Alda-1 on  $V_{\max}$  was not dependent on the structure of the aldehyde substrate, it can be estimated that the drug decreased the binding affinity of formaldehyde  $\sim 20$ -fold. However, this model leaves unexplained the comparably small effect of Alda-1 on oxidation of the longer chain propionaldehyde, pointing at additional as yet unknown factors that may govern the effects of Alda-1.

**Effects of Alda-1 on Ester Hydrolysis**—In the absence of NAD, Alda-1 caused 6- and 9-fold increases in the rates of ester hydrolysis catalyzed by ALDH2\*1 and ALDH2\*2, respectively (Fig. 4A). Intriguingly, the degree of enzyme stimulation by Alda-1 was virtually identical to that exerted by NAD, as if the drug mimicked the effect of the coenzyme on ester hydrolysis. The seemingly aberrant data obtained in the combined presence of Alda-1 and NAD are explained by a biphasic effect of NAD on esterase activity, resulting in pronounced inhibition at

high concentrations of the coenzyme (Fig. 4B). Inhibition of ALDH2 by high NAD has been observed previously (28, 39) and was suggested to result from competition for *p*-NPA binding (28). However, a more recent paper reported on monophasic stimulation of both ALDH2 variants by NAD (22). It was beyond the scope of the present study to clarify this issue, but obviously the pronounced left shift of NAD-induced inhibition of wild type esterase activity by Alda-1 provides a conclusive explanation for the divergent effects of the drug on the two ALDH2 variants that we observed in the presence of 1 mM NAD (Fig. 4).

**Effects of Alda-1 on GTN Denitration**—Surprisingly, Alda-1 did not stimulate but inhibited GTN denitration by both ALDH2 variants (Fig. 5). Considering that the yield of GTN-derived products is determined by the balance between the rates of GTN denitration and mechanism-based enzyme inactivation (30, 40), it appears that the increased rate of ALDH2 inactivation observed in the presence of Alda-1 counteracts and, in fact, overcompensates the stimulation of the enzyme that would be expected from the esterase data. It was one of the goals of the present study to find out whether Alda-1 might be useful as a lead compound for the development of drugs improving the potency of GTN in subjects carrying the ALDH2\*2 allele, but our data clearly argue against this possibility.

Taken together, our results indicate that Alda-1 modifies ALDH2 function mainly through increasing the apparent binding affinity for NAD, which is essential for aldehyde oxidation and facilitates ester hydrolysis as well as GTN denitration. This effect is particularly pronounced in the case of the Asian variant, which exhibits very low NAD affinity. Protein crystallography revealed profound structural disorders in the active site of ALDH2\*2 (21, 22). Thus, Alda-1 may restore the active site conformation, resulting in increased NAD binding affinity and, as a consequence, increased dehydrogenase and esterase activities. In addition, Alda-1 appears to allosterically reduce the binding affinity of aldehyde substrates, resulting in inhibition of dehydrogenase activity measured at subsaturating aldehyde concentrations.

**GTN Bioactivation**—Although the E487K substitution led to  $\sim 7$ -fold lower rates of GTN denitration as compared with wild type, the rate of NO formation was reduced only 2-fold. This discrepancy reinforces our recent proposal that GTN denitration and bioactivation reflect two separate pathways of ALDH2-catalyzed GTN biotransformation, both of which involve formation of a thionitrate intermediate at Cys<sup>302</sup> as initial reaction step (12). The factors governing the fractional contribution of these two putative pathways to overall GTN turnover as well as the underlying molecular mechanisms are unknown and are currently being investigated in our laboratory.

The slower decay of the NO signal observed with ALDH2\*2 in the presence of SOD may reflect a lower rate of mechanism-based enzyme inactivation by GTN (Table 2). This hypothesis is supported by a comparison of the observed inactivation rate constants (Table 2) and the apparent rate constants for NO decay calculated from the data shown in Fig. 3A. As elaborated under "Experimental Procedures," there should be a close rela-

relationship between the respective rate constants, with  $k_{\text{obs}} = 0.5\text{--}1.0 \times k_{\text{inact}}$  if decay of the NO signal reflects ALDH2 inactivation, although the apparent inactivation rate constant was presumably decreased by DTT (30) and increased by the higher temperature (37 versus 25 °C) in the electrochemical measurements of NO formation as compared with the enzyme inactivation studies. Notwithstanding these caveats, the ratio of the rate constants observed with the two variants of the enzyme would be expected to be similar. The rates of ALDH2\*1 and ALDH2\*2 inactivation were  $8.2 \pm 0.44 \times 10^{-3} \times \text{s}^{-1}$  and  $2.5 \pm 0.24 \times 10^{-3} \times \text{s}^{-1}$ , respectively (Table 2), yielding a ratio of  $3.3 \pm 0.36$ . First order decay of the NO signals obtained with ALDH2\*1 and ALDH2\*2 gave values of  $3.8 \pm 0.02 \times 10^{-3} \times \text{s}^{-1}$  and  $1.1 \pm 0.02 \times 10^{-3} \times \text{s}^{-1}$ , respectively (assuming  $k_{\text{obs}} = 0.5 \times k_{\text{inact}}$ ), which yields a ratio of  $3.4 \pm 0.05$ . This remarkable concordance strongly suggests that the prolonged NO signal obtained with the ALDH2\*2 (despite lower initial rates of NO formation) reflects the comparably slow rate of GTN-induced inactivation of the Asian mutant. If inactivation of vascular ALDH2 were indeed a major cause of nitrate tolerance, these results would imply delayed tolerance development in subjects carrying the mutation.

In the sGC bioactivation assay the E487K mutant was less efficient than wild type at 1  $\mu\text{M}$  GTN (Fig. 6B) as expected from the lower apparent affinity for the nitrate observed in the denitration assays (Fig. 1A). However, under conditions of high turnover, *i.e.* saturating GTN (Figs. 2A and 6A) and/or high protein concentrations (Fig. 2B), the Asian variant had the opposite effect, resulting in higher rates of cGMP formation than the wild type enzyme. Because sGC activation by both variants was virtually identical in the presence of the superoxide scavenger SOD (data not shown), the higher efficiency of ALDH2\*2 in the absence of SOD appears to reflect reduced superoxide generation and consequently increased NO bioavailability in the course of ALDH2\*2-catalyzed GTN turnover. The biological relevance of mechanism-based superoxide formation in the course of GTN bioconversion is unclear. Although high amounts of manganese-SOD present in mitochondria may efficiently prevent superoxide-mediated inactivation of NO, superoxide scavenging was proposed to be compromised in nitrate-tolerant blood vessels (41).

Regardless of the *in vivo* relevance of ALDH2-catalyzed superoxide formation, the differences between wild type and the E487K mutant are interesting with respect to enzyme function. In a recent study we characterized an ALDH2 mutant lacking the general base glutamate 268 (E268Q) (12). In the absence of NAD, the E268Q mutant exhibited similar rates of GTN denitration as the wild type enzyme, but surprisingly generation of superoxide occurring in the course of GTN denitration was reduced, resulting in increased NO bioavailability and enhanced sGC activation in the absence of SOD. Remarkably, one of the structural changes of the E487K substitution in ALDH2\*2 involves relocation of the carboxylate group of Glu<sup>268</sup> 5.5 Å away from the sulfhydryl group of the essential nucleophilic residue Cys<sup>302</sup> (22). Thus, it is tempting to speculate about disturbance of the Glu<sup>268</sup>-Cys<sup>302</sup> interaction as a common cause of the increased NO bioavailability that we

observed in the course of GTN bioactivation by E268Q-ALDH2 and the Asian variant.

*Implications for GTN Vasoactivity*—Extrapolation of our data obtained with purified enzymes to the *in vivo* situation would imply that the vascular response to low doses of GTN should be reduced in subjects carrying the ALDH2\*2 allele, whereas the response to high doses should be preserved. Two clinical studies in which this issue has been addressed provide strong evidence for decreased efficacy of intra-arterially infused (23) and sublingually administered (24) GTN in subjects carrying the ALDH2\*2 allele. In one study, the E487K substitution impaired the change in blood flow of healthy volunteers at all of the GTN infusion rates tested (0.25–4.0  $\mu\text{g}/\text{min}$ ), but comparison with the effect of a high dose of the ALDH2 inhibitor disulfiram suggests that ALDH2 still contributes significantly to vasodilation induced by high GTN in carriers of the ALDH2\*2 allele (*cf.* Tables 2 and 3 in Ref. 23). Another study reported that the efficacious rate of sublingual GTN, measured as reduction of ischemic chest pain of patients having documented coronary artery disease, was reduced from 85.1% in controls to 57.6% in subjects carrying at least one ALDH2\*2 allele (24). Based on these data and the assumption that ALDH2\*2 exhibits very low activity, the authors concluded that factors other than ALDH2 may contribute to GTN efficacy in carriers of the ALDH2\*2 allele. The issue may be even more complex because higher rates of protein turnover result in lower protein expression levels of the Asian variant as compared with wild type (42). Moreover, in a recent study carried out by Dr. John D. Horowitz and co-workers in a blinded fashion with 51 young Chinese adults, the reduction in the hemodynamic response to sublingual GTN was very small and significant only in carriers of the ALDH2\*2 allele.<sup>3</sup> Thus, there is considerable evidence that subjects expressing the E487K mutant are still responsive to GTN. The present results showing that the Asian variant catalyzes significant bioactivation of GTN despite very low denitration activity may at least partially explain the GTN response in these subjects.

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