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Mutation of Arg-115 of human class III alcohol dehydrogenase: A binding site required for formaldehyde dehydrogenase activity and fatty acid activation

(site-directed mutagenesis/kinetic characterization/mutant enzymes/substrate specificity/enzyme activation)

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ABSTRACT **The origin of the fatty acid activation and formaldehyde dehydrogenase activity that distinguishes human class III alcohol dehydrogenase (alcohol:NAD$^+$ oxidoreductase, EC 1.1.1.1) from all other alcohol dehydrogenases has been examined by site-directed mutagenesis of its Arg-115 residue. The Ala- and Asp-115 mutant proteins were expressed in *Escherichia coli* and purified by affinity chromatography and ion-exchange HPLC. The activities of the recombinant native and mutant enzymes toward ethanol are essentially identical, but mutagenesis greatly decreases the k_{cat}/K_m values for glutathione-dependent formaldehyde oxidation. The catalytic efficiency for the Asp variant is <0.1% that of the unmutated enzyme, due to both a higher K_m and a lower k_{cat} value. As with the native enzyme, neither mutant can oxidize methanol, be saturated by ethanol, or be inhibited by 4-methylpyrazole; i.e., they retain these class III characteristics. In contrast, however, their activation by fatty acids, another characteristic unique to class III alcohol dehydrogenase, is markedly attenuated. The Ala mutant is activated only slightly, but the Asp mutant is not activated at all. The results strongly indicate that Arg-115 in class III alcohol dehydrogenase is a component of the binding site for activating fatty acids and is critical for the binding of *S*-hydroxymethylglutathione in glutathione-dependent formaldehyde dehydrogenase activity.**

Class III alcohol dehydrogenase ($\chi\chi$-ADH; native dimeric class III alcohol dehydrogenase isolated from human liver) is catalytically unique among the human alcohol dehydrogenases (ADHs; alcohol:NAD$^+$ oxidoreductase, EC 1.1.1.1). Although it is homologous with the other enzymes of the ADH family (1) and, like them, a dimeric enzyme with an essential zinc at the active site, its kinetic properties differ markedly (2). It is the only ADH identified thus far that is capable of oxidizing formaldehyde in a glutathione (GSH)-dependent reaction (3, 4). Moreover, its K_m for ethanol is >3 M compared to 1 mM for class I isozymes; yet, it is nearly as effective as other ADHs toward long-chain primary alcohols, particularly ω-hydroxy fatty acids such as 12-hydroxydodecanoate (12-HDA) (5). It is totally insensitive to inhibition by 4-methylpyrazole, a potent inhibitor of the class I and II enzymes.

An additional feature unique to $\chi\chi$-ADH is its activation by hydrophobic anions, particularly fatty acids, which can stimulate both alcohol oxidation and aldehyde reduction up to 30-fold (5). The nonessential activator mechanism proposed for this process indicates that the alcohol substrate and the activator bind simultaneously, with consequent enhancement of substrate binding. The degree of activation is optimal when the sum of the fatty acid plus alcohol chain lengths is 9 or 10 carbons. Thus, ethanol oxidation is activated maximally by octanoate, pentanol by pentanoate, and octanol by acetate. This complementarity likely reflects the length of the substrate binding pocket of the ADHs (6, 7). Appropriate combinations of activator and alcohol side chains apparently fit into this pocket (5) and result in maximal activity. The observations that all activators require an anionic group and that the best substrates for $\chi\chi$-ADH, 12-HDA and *S*-hydroxymethylglutathione (HMGSH), have an ω-carboxylate group suggested that a corresponding positively charged residue would be located close to the active site. The most likely candidate is Arg-115, which recently has been implicated as a binding locus for both activators and HMGSH: its modification by phenylglyoxal diminishes both fatty acid activation and formaldehyde dehydrogenase (FDH) activity (J.-M. Moulis, B.H., K.E., and B.L.V., unpublished data), and it is located in this region of the enzyme. The charged nature of the substrate binding site of class III ADH has also been noted in sequence comparisons and computer graphics modeling (6). When an activator binds to fill the region of the pocket not occupied by the substrate, its carboxylate group would interact with this positively charged arginine. This model could also account for the FDH activity of $\chi\chi$-ADH since oxidation of HMGSH, the actual substrate formed spontaneously from GSH and formaldehyde, would be facilitated by interaction of one of its two carboxyl groups with Arg-115, thereby aligning its primary hydroxyl with the active site zinc.

Here we report the kinetic characterization of human class III ADH altered at position 115 by *in vitro* mutagenesis to Asp and to Ala. Comparison of these mutant enzymes with r$\chi\chi$-ADH (recombinant dimeric class III ADH expressed in *Escherichia coli*) shows that Arg-115 is required for both the high formaldehyde oxidizing activity and the activation by hydrophobic anions. The results support a common basis for both properties.

MATERIALS AND METHODS

Plasmid Constructions. The entire coding cDNA for human class III ADH was excised from plasmid p30L (8) with *Eco*RI and *Ssp* I and was expressed (M.E., unpublished data) in the same system that was used for class I ADH (9, 10). Mutagenesis of Arg-115 to Ala was performed by an overlap

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Abbreviations: ADH, alcohol dehydrogenase; $\chi\chi$-ADH, native dimeric class III ADH isolated from human liver; r$\chi\chi$-ADH, recombinant dimeric class III ADH expressed in *E. coli*; FDH, formaldehyde dehydrogenase; 12-HDA, 12-hydroxydodecanoic acid; GSH, glutathione; HMGSH, *S*-hydroxymethylglutathione; MCA, methylcrotyl alcohol.

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extension PCR method (11). Primers were constructed to create a unique *Stu* I site together with the desired mutation in a *Dra* III/*Age* I fragment of the p30L clone (J.-M. Moulis, B.H., K.E., and B.L.V., unpublished data). This mutated fragment was then used to replace the corresponding part of the cDNA in the expression vector. Mutation of Arg-115 to Asp was performed on a 393-bp *Eco*RI/*Kpn* I fragment that was subcloned into M13mp18. Single-stranded DNA was isolated and the mutation was performed with a 21-mer mismatching oligonucleotide (12). Sequences of mutated fragments were checked by dideoxynucleotide sequence analysis (13) for the presence of the correct mutations and to ensure that other mutations had not occurred.

Expression and Protein Purification. r$\chi\chi$-ADH and the Arg-115 to Ala (R115A) and Arg-115 to Asp (R115D) mutants were expressed in 2-liter cultures of *E. coli* strain TG1 or XL1-Blue, both *lacI*^{q}, after induction with 0.3 mM isopropyl β-D-thiogalactopyranoside. Cells were harvested and disrupted in 1 mM dithiothreitol/10 mM Tris·HCl, pH 8, by sonication before centrifugation for 60 min at 48,000 × *g*. The supernatant, ≈20 ml, was applied to a 150-ml DEAE column (DE-52; Whatman). The unretained fraction containing the enzyme, ≈50 ml, was applied to a column (1 × 10 cm) of AMP-Sepharose (Pharmacia). The column was washed with 5 vol of 100 mM Tris·HCl (pH 8) containing 0.5 mM dithiothreitol and the recombinant enzymes eluted with 1 mM NAD^+ added to the wash buffer. Fractions exhibiting activity toward ethanol were concentrated with an Amicon concentrator equipped with a PM 30 membrane and exchanged into 10 mM Tris·HCl, pH 8.0/1 mM dithiothreitol. Final purification was by HPLC on a DEAE-5PW (Waters) anion-exchange column under conditions previously established for the liver enzyme (5). Protein concentrations were measured with Coomassie brilliant blue (Pierce) with bovine serum albumin as a standard. Protein purity was checked by SDS/PAGE and UV spectra were recorded for all isolated proteins on a HP 8451 spectrophotometer.

Enzyme Activity. Enzymatic activity was determined by following NADH formation at 340 nm (ε = 6.22 $mM^{-1}\cdot cm^{-1}$) with a Beckman DU 7500 spectrophotometer. Oxidation of ethanol, octanol, and 12-HDA was measured in 0.1 M glycine/NaOH, pH 10, and that of methylcrotyl alcohol (MCA) was measured in 50 mM sodium phosphate (pH 8.5) (5). FDH activity toward HMGSH was measured in sodium phosphate (pH 8) with 1 mM GSH and 1 mM formaldehyde, except where otherwise indicated. An NAD^+ concentration of 2.4 mM was used in all measurements. One unit of enzyme is the amount that produces 1 μmol of NADH per min. Alcohols of analytical grade were used without further purification and formaldehyde solutions were prepared from newly opened glass ampules containing 20% solutions (Ladd Research Industries, Burlington, VT). Octanol, 12-HDA, and MCA were all dissolved in methanol to yield a final assay mixture containing 3.3% methanol, a methanol concentration that does not significantly affect $\chi\chi$-ADH activity. Inhibition studies with 4-methylpyrazole were performed with 0.5 M ethanol and the mixtures were preincubated without substrate for 10 min at room temperature before addition of enzyme to initiate the reaction. A weighted non-linear-regression analysis program was used to calculate kinetic parameters (14). Modifications with phenylglyoxal were in 0.1 M sodium bicarbonate, pH 8.1/1 mM phenylglyoxal, at 23°C. Reactions were allowed to proceed for 40 min and aliquots were withdrawn and assayed with HMGSH (1 mM GSH/0.1 and 1 mM formaldehyde) and with 0.1 mM MCA. Activation of MCA oxidation was measured in the presence of pentanoate (in 50 mM sodium phosphate adjusted to pH 8.5) at a final concentration of up to 50 mM.

RESULTS

Expression and Purification of ADH Mutants. Three forms of human class III ADH have been expressed in *E. coli*: R115A, R115D, and r$\chi\chi$-ADH. The latter was required for comparison since $\chi\chi$-ADH isolated from human liver is acetylated at the N terminus (15), whereas the recombinant enzymes are not (9). Anion-exchange HPLC was used as the final purification step (5) to obtain homogeneous products with a yield of ≈1 mg of protein per 2 liters of culture. These eluted at slightly different retention times (Table 1) in accord with the changes in charge introduced at position 115. Thus, r$\chi\chi$-ADH elutes earlier than $\chi\chi$-ADH, consistent with its unblocked N terminus and the Asp mutant elutes somewhat later owing to the introduction of a negative charge. SDS/PAGE (data not shown) indicates that all of the enzymes are >95% homogeneous.

Kinetic Characterization. The kinetic effects of the mutations were assessed with both neutral and charged alcohols and with HMGSH generated from GSH and formaldehyde (Table 2). For all substrates examined, the kinetic constants for r$\chi\chi$-ADH are essentially the same as those of $\chi\chi$-ADH. All quantitative comparisons of kinetic parameters are made with the recombinant enzyme. None of the three recombinant forms, with Arg, Ala, or Asp at position 115, could be saturated with ethanol as substrate. Plots of v versus [S] were linear up to 3 M ethanol (data not shown) and, hence, only k_{cat}/K_m values could be calculated (Table 2). Their specific activities toward ethanol are all roughly comparable, although the Asp and Ala mutants are ≈1/3rd less active. As with the native enzyme, methanol is not a substrate for either of the mutants nor are they inhibited by 4-methylpyrazole up to 5 mM, indicating that they have not acquired class I ADH characteristics.

Long-chain alcohols are considerably better substrates for the class III enzymes than is ethanol (2, 5, 16), and this is true also for both mutants. Mutagenesis produces only minor changes in the K_m for octanol but much greater effects in that for 12-HDA and HMGSH. The results of mutation on k_{cat} are relatively small for R115A and are somewhat greater for R115D. The combined effect of these changes is to decrease k_{cat}/K_m for 12-HDA by a factor of 28 for R115A and by a factor of 67 for R115D. With HMGSH, the 70-fold increase in K_m and a 2-fold decrease in k_{cat} results in an overall 150-fold decrease in k_{cat}/K_m for R115A. The decrease of catalytic activity is even more pronounced for R115D, where K_m increases 228-fold and k_{cat} is lowered markedly for an overall decrease in k_{cat}/K_m of >1300-fold (Table 2).

Effects on Activation by Pentanoate. Under conditions where $\chi\chi$-ADH is activated 12-fold (5), pentanoate activates the MCA activity of r$\chi\chi$-ADH 10-fold (Fig. 1). In contrast, R115A was activated only ≈2-fold and R115D was not activated at all by up to 50 mM pentanoate. K_m and k_{cat} values of the mutant enzymes with MCA as substrate were not significantly changed compared to those for $\chi\chi$-ADH (data not shown).

Table 1. HPLC retention times of $\chi\chi$-ADH, r$\chi\chi$-ADH, and mutant enzymes

Protein	Retention time, min	Charge change
$\chi\chi$-ADH	13.53	—
r$\chi\chi$-ADH	12.47	+1
R115A	12.81	0
R115D	13.88	−1

HPLC conditions are those from ref. 5 at pH 8.0. The change in charge is due to an unblocked α-amino group and the side-chain replacement at residue 115. The actual charge change will be affected by the pK of the α-amino group.

Table 2. Kinetic parameters of $\chi\chi$-ADH, r$\chi\chi$-ADH, R115A, and R115D

Substrate	$\chi\chi$-ADH	r$\chi\chi$-ADH	R115A	R115D
		K_m, μM		
Octanol	2000	2200	1200	2200
12-HDA	60*	74	680	1300
HMGSH	4†	4	280	910
		k_{cat}, min^{-1}		
Octanol	440	360	150	240
12-HDA	170*	370	122	97
HMGSH	200†	210	97	36
		k_{cat}/K_m, $mM^{-1}\cdot min^{-1}$		
Ethanol	0.045†	0.045	0.015	0.015
Octanol	220	160	125	110
12-HDA	2,800*	5,000	180	75
HMGSH	50,000†	53,000	350	40

*From ref. 2.
†From ref. 4.

Modification by Phenylglyoxal. Phenylglyoxal modification of r$\chi\chi$-ADH under conditions previously used with $\chi\chi$-ADH (J.-M. Moulis, B.H., K.E., and B.L.V., unpublished data) produced identical results: a complete loss of activation with fatty acids, loss of >90% of FDH activity, and an ≈3-fold increase in activity with MCA as substrate. Similar treatment of both R115A and R115D had no effect on the MCA or the FDH activity of either enzyme.

DISCUSSION

The role of Arg-115 of $\chi\chi$-ADH in binding HMGSH and fatty acid activators has been examined with two mutants of the enzyme, R115D and R115A. The experiments have served to establish a common basis for two properties that are unique to class III ADHs—namely, the ability to be activated by fatty acids (5) and the GSH-dependent FDH activity (3, 4). Chemical modification of $\chi\chi$-ADH with phenylglyoxal had indicated that Arg-115 is involved in these two class III ADH-specific properties (J.-M. Moulis, B.H., K.E., and B.L.V., unpublished data). We therefore decided to characterize two human class III ADH proteins mutated at this position. Asp was chosen to replace Arg-115 since it is the residue at position 115 in all mammalian class I isozymes, and none of these is activated by fatty acids nor does any exhibit FDH activity. Furthermore, this substitution completely inverts the charge at this site and allows us to test the hypothesis that the anionic groups of ω-carboxylate substrates, the hemithiolacetal HMGSH, and fatty acid activators interact at this position. The Ala mutant was chosen to examine the effect of removing the charge at position 115.

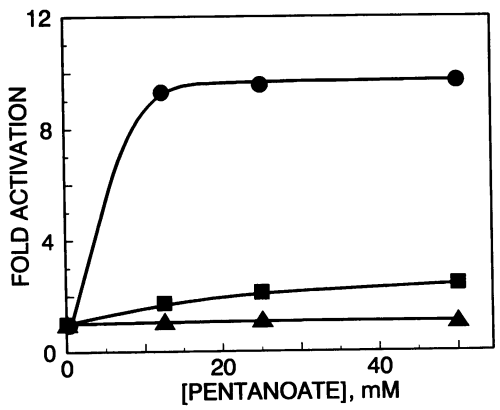

FIG. 1. Effect of pentanoate on oxidation of MCA by r$\chi\chi$-ADH (●), R115A (■), and R115D (▲).

Expression of r$\chi\chi$-ADH and its two mutants, R115D and R115A, in *E. coli* was accomplished with the system devised for the class I isozymes (10) and other human class III ADH mutants (M.E., unpublished data). The activities of the two mutants toward ethanol were essentially unchanged (Table 2) compared to $\chi\chi$-ADH, and their behavior during purification was consistent with the changes made in amino acid composition. The absence of acetylated N termini of the recombinant proteins as well as the mutations from the positively charged Arg to Ala and Asp affect the total charge of the protein and, hence, their respective elution times from the HPLC ion-exchange column (Table 1). Furthermore, affinity chromatography on AMP-Sepharose indicates that in both cases the coenzyme binding site is intact and, hence, that this part of the enzymes has folded correctly. Although *E. coli* produces an endogenous class III alcohol dehydrogenase homologous to the human enzyme (17), the *E. coli* enzyme has a pI of 4.4 compared to 6.4 for the human protein (2), and it would be well separated from any of the mutants; indeed, it was not observed in these preparations. The final products after HPLC were essentially homogeneous by SDS/PAGE and migrated to the same extent as the human liver enzyme monomers with a molecular mass of 40 kDa.

Changing Arg-115 to Asp alters the substrate specificity of $\chi\chi$-ADH as reflected in the loss of catalytic efficiency (k_{cat}/K_m) for GSH-dependent formaldehyde oxidation essentially without any effect on activity toward the neutral alcohols ethanol, MCA, and octanol. This change in specificity is the result of markedly increased K_m values for anionic substrates, such as the hemithiolacetal of GSH. The specific activities and k_{cat}/K_m values of r$\chi\chi$-ADH and its mutants toward ethanol are essentially identical to those of $\chi\chi$-ADH (Table 2), and thus position 115 does not appear to be directly involved in ethanol oxidation. Similarly for octanol, there are only slight changes in k_{cat} and K_m between the mutants and the native enzyme. In contrast, with HMGSH and 12-HDA there is clear evidence for a significant role for Arg-115 in substrate binding. K_m is raised 9- and 18-fold toward 12-HDA with the Ala and Asp mutants, respectively. With HMGSH the change is even more dramatic: the corresponding increases are 70- and 230-fold, respectively. The greatest change in k_{cat} is also with the Asp mutant toward HMGSH with a >5-fold decrease compared to the r$\chi\chi$-ADH. In general, the data demonstrate that the substantially decreased activity of the mutated enzymes toward substrates bearing carboxyl groups is mainly attributable to a drastic change in binding constants.

The abolition of both GSH-dependent FDH activity and fatty acid activation by modification of class III ADH with phenylglyoxal had pointed to a role for Arg-115 in both processes. Class III ADH is the only one of the human ADHs with an Arg residue at position 115 (Table 3): class I and class II ADHs have Asp and Ser, respectively, in this position and are devoid of FDH activity and fatty acid activation. Human liver class III ADH, but not the other human ADHs, also has Arg residues at positions 231, 276, and 304. However, these locations are all far removed from the active site (6) and are unlikely to be involved in enzyme activity or fatty acid activation. This can be judged from computer modeling studies, which have shown that the main chain of class III ADH can be built without major changes to the known crystal structure of the horse EE isozyme and which allow distances between residues to be determined (6). Several plant ADHs also have an Arg at position 115 (Table 3), but it is not known whether these ADHs have FDH activity or undergo fatty acid activation. Thus far, within the mammalian ADHs FDH activity, fatty acid activation, and an Arg at position 115 are unique features of class III (Table 3).

Functionally, class III ADH is the only one (*i*) that cannot be saturated by ethanol, (*ii*) that is activated by fatty acids (5),

Table 3. Comparison of sequences of ADHs around residue 115

	115
Human class III	LCQKIRVTQGK
Horse class III	LCQKIRTTQGK
Rat class III	LCQKIRVTQGK
Maize 1	MCDLLRINTDR
Maize 2	MCDLLRINVDR
Pea	MCDLLRINTDR
Arabidopsis	MCDLLRINTER
Human class I α	YCLKNDVSNPQ
Human class I β	YCLKNDLGNPR
Human class I γ	YCLKNDLGNPR
Human class II	LCGKISNLKSP
Human class V	FCIQFKQSKTQ
Chicken liver	LCIKNDLSSSP
Cod class I	QCVKGWANESP
Horse class I E	FCLKNDLSMPR

Sources for the sequences are as follows: human class III, ref. 1; rat and horse class III, ref. 18; human class V, ref. 19; chicken liver, ref. 20; cod class I, ref. 21; all others, ref. 22.

and (*iii*) whose substrate specificity includes hemithiolacetals generated from GSH and other thiols (ref. 4; B.H. and B.L.V., unpublished data). Its sequence is highly conserved with almost 95% positional identity between human and rat compared to 82% for the class I ADHs (1). A class III ADH has also been identified in *E. coli*, where no typical class I ADH has been detected. *E. coli* ADH exhibits a high degree of residue identity, ≈60%, within the 57 N-terminal residues sequenced compared to other known χχ-ADHs (17). ADHs have also been traced in the piscine line, and an enzyme with characteristics of class I ADH was recently isolated (21). However, in terms of structure this latter enzyme is more closely related to class III than to class I ADH. This strongly suggests that the class III type of ADH is the evolutionary precursor of the diverged mammalian ADHs (23). FDH activity apparently emerged early on the evolutionary scale to detoxify formaldehyde by the oxidation of its conjugate with GSH. Gene duplication and subsequent loss of the anion binding site, Arg-115, as ADH classes diverged, together with other changes, resulted in loss of FDH activity and fatty acid activation and enhancement of short-chain alcohol oxidation. The latter has been ascribed to additional mutations, primarily in the size and hydrophobicity of the active site pocket, which improve the capacity to bind short-chain alcohols and, in particular, to oxidize ingested ethanol more effectively (6). The capacity to metabolize short-chain alcohols by class III ADH is very low owing to poor substrate binding and has been acquired by the other classes that diverged from this early enzyme.

In summary, mutagenesis of human class III ADH has identified a residue, Arg-115, that is largely responsible for two of its unique functional properties, the capacity to oxidize HMGSH and the activation by fatty acids. The results lead to a common mechanism that explains both of these activities. Although not a catalytic residue, Arg-115 plays a definitive role in establishing the specificity of this ADH. It allows an ω-carboxy-substituted hydrophobic moiety, be it from the substrate or an activator, to achieve full occupancy of the active site pocket, which thereby increases the catalytic efficiency of the χχ-ADH oxidation process. The optimal size of activator/substrate pairs for fatty acid activation and of hemithiolacetals for FDH activity is dictated by the dimensions of the active site pocket. The preference of the enzyme for long, hydrophobic anionic substrates; the increase in activity toward MCA; and loss of both FDH activity as well as activation by fatty acids when the arginine is blocked by modification by phenylglyoxal are all consistent with the requirement for a positively charged residue at position 115.

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