Role of Arginine 115 in Fatty Acid Activation and Formaldehyde Dehydrogenase Activity of Human Class III Alcohol Dehydrogenase†

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ABSTRACT: Modification of class III alcohol dehydrogenase (χχ-ADH) with phenylglyoxal eliminates fatty acid activation by pentanoate and octanoate and concomitantly increases specific activity toward ethanol and 3-methylcrotyl alcohol 2–3-fold. In contrast, chemical modification decreases activity toward *S*-(hydroxymethyl)glutathione (FDH activity) and 12-hydroxydodecanoic acid by increasing K_m, pointing to a role for arginine in binding anionic substrates. Modification with [7-^{14}C]phenylglyoxal indicates that only one arginine residue per subunit is modified. Sequence analysis of tryptic peptides indicates that Arg-115 is modified. Site-directed mutation of this residue to alanine eliminates both fatty acid activation and FDH activity, thus confirming the identity of the modified residue and its function. These results account in part for the unique specificity of χχ-ADH relative to other human ADH isozymes.

Among the human alcohol dehydrogenase (ADH)[1] isozymes, χχ-ADH (class III) is unique with regard to tissue distribution, substrate specificity, and sensitivity to inhibitors [reviewed in Vallee and Bazzone (1983)] as well as response to activators (Moulis et al., 1991). First recognized as the only ADH isozyme in human placenta (Parés & Vallee, 1981), χχ-ADH occurs in most tissues, but in addition is the only form in brain and testis (Beisswenger et al., 1985). Its sequence is 82% identical to that of other human ADHs (Kaiser et al., 1991), yet it is the only isozyme that both is activated by fatty acids (Moulis et al., 1991) and oxidizes formaldehyde (Koivusalo et al., 1989; Holmquist & Vallee, 1991); i.e., it catalyzes the oxidation of *S*-(hydroxymethyl)glutathione (HMGSH), the spontaneous adduct of formaldehyde and glutathione, to the thioformate ester of GSH.

Activation by fatty acids and FDH activity both require an anionic group located at a suitable distance from the alcohol or thiohemiacetal function of the substrate. Anion activation, as high as 30-fold, follows a nonessential mechanism and is observed only with anionic hydrocarbons, chiefly fatty acids (Moulis et al., 1991). Neutral or positively charged hydrophobic compounds are ineffective. Similarly, anionic thiohemiacetals can serve as substrates (Holmquist & Vallee, 1991); the best, such as HMGSH and the hemithioacetal of 6-mercaptohexanoic acid, as well as 12-hydroxydodecanoic acid, contain a negatively charged carboxylate 8–10 carbon atoms removed from the hydroxyl group. These observations suggested that the substrate binding pocket of χχ-ADH might contain a cationic group, either arginine or lysine, responsible for the specific anion recognition. We have therefore examined the effects of chemical modification with lysine- and arginine-specific reagents and have found that phenylglyoxal selectively modifies Arg-115 and markedly curtails both fatty acid activation and FDH activity without significantly affecting ethanol dehydrogenase activity. These results point to a critical role for Arg-115 as an important anion binding locus in χχ-ADH.

MATERIALS AND METHODS

Materials. Human liver class III ADH purification and kinetic analysis (Moulis et al., 1991) and human class I isozyme isolation (Wagner et al., 1983) and purification (McEvily et al., 1991) were as previously described. A horse liver ADH suspension (Boehringer Mannheim, FRG) was solubilized as described (Maret et al., 1979). Prior to modifications, the horse and human class I isozymes were freed of bound cofactor with Affi-gel Blue (Bio-Rad, Richmond, CA) affinity resin (McEvily et al., 1991). Protein concentrations were measured by the method of Lowry (1951) except as noted. Phenylglyoxal monohydrate (Aldrich Chemical Co., Milwaukee, WI) was recrystallized from hot water and kept in the dark under nitrogen. All other reagents were used as supplied by the manufacturer.

Chemical Modifications. Reductive methylation (Jentoft & Dearborn, 1981) was performed on enzymes that had been exchanged into 50 mM Hepes, pH 7.5, by repeated dilution and concentration in a Centricon 30 microconcentrator (Amicon, Danvers, MA) to a final isozyme concentration of 2.5 μM. The time course of reaction with formaldehyde, 1.5 mM, and sodium cyanoborohydride, 7.5 mM, at 20 °C was followed after removal of these reagents from samples by ultrafiltration and by measuring enzymatic activity with 33 mM ethanol as substrate for human class I and horse isozymes and 0.5 M ethanol as substrate for human class III ADH.

A fresh 10 mM solution of phenylglyoxal in 0.1 M sodium bicarbonate buffer, pH 8.1, was added to χχ-ADH in the same buffer at 25 °C. Changes in enzymatic activity were followed by direct addition of 10-μL samples to 1-mL assay mixtures as previously described (Moulis et al., 1991; Holmquist & Vallee 1991) with 3-methylcrotyl alcohol (MCA) and HMGSH as substrates, unless otherwise indicated. 12-Hydroxydodecanoic acid (12-HDA) oxidation was measured

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[1] Abbreviations: ADH, alcohol dehydrogenase; χχ-ADH, homodimeric class III ADH composed of χ-ADH subunits; rχχ-ADH, recombinant class III ADH; R115A, rχχ-ADH in which Arg-115 is replaced by Ala; FDH, formaldehyde dehydrogenase; HMGSH, *S*-(hydroxymethyl)-glutatione; MCA, 3-methylcrotyl alcohol, 3-methyl-2-buten-1-ol; 12-HDA, 12-hydroxydodecanoic acid; NEM, *N*-ethylmorpholine; DTT, dithiothreitol; NAD$^+$, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid.

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at pH 10 in 0.1 M glycine, 1 mM substrate, and 2.4 mM NAD^+. Activity toward ethanol was measured at pH 10 in 0.1 M glycine and 2.4 mM NAD^+ with 0.5 M substrate. One unit of activity is the amount of enzyme required to convert 1 μmol of NAD^+ to NADH in 1 min. Fatty acid activation was assessed by addition of 100 μL of 0.5 M pentanoate to the MCA assay mixture. For incorporation of [7-^{14}C]-phenylglyoxal (Amersham, Arlington Heights, IL) into χχ-ADH, 1.7 mg of enzyme was incubated in 0.1 M sodium bicarbonate, pH 8.1, containing 1 mM labeled phenylglyoxal. After 1 h, the reaction was stopped by gel filtration on a PD-10 column (Pharmacia, Piscataway, NJ) equilibrated with 50 mM phosphate buffer, pH 5.8. Incorporation of radiolabel was determined by adding 10 μL of enzyme solution to 3.5 mL of Econofluor (NEN, Boston, MA) and measuring radioactivity with a Beckman LS 1801 liquid scintillation counter.

Isolation of Labeled Peptides. All steps were performed in a Centricon 30 ultrafilter. Phenylglyoxal-labeled enzyme from the gel chromatography step was first concentrated to 100 μL, denatured by addition of 400 μL of 6 M guanidine hydrochloride in 0.1 M *N*-ethylmorpholine (NEM) and 1 mM EDTA, pH 8.5, and concentrated again to 100 μL. It was then carboxymethylated with 20 mM iodoacetamide in 5 mM NEM, pH 8.5, under nitrogen for 1 h, washed 3 times with 0.1 M NEM, pH 8.5, and concentrated to 100 μL. The sample was digested by addition of 40 μL of 1 mg/mL trypsin and incubated at 36 °C for 4 h. The reaction was terminated by addition of 350 μL of buffer A (0.1 M NaOH, 0.1 M perchloric acid, and 0.1% phosphoric acid, pH 2.5), and the peptides were collected by ultrafiltration. The retentate was washed with an additional 350 μL of buffer A, and the combined ultrafiltrates were lyophilized. The peptides were dissolved in 220 μL of buffer A; a 10–μL sample was removed for radioactivity measurement, and the rest was chromatographed on a Waters Nova-Pak C_{18} HPLC column (3.9 × 150 mm) at 1 mL/min with the following gradient schedule: buffer A diluted with buffer B (prepared by dilution of buffer A 1:4 with acetonitrile), initial 98%; 70 min, 62%; 130 min, 20% with linear segments. Fractions (1.5 mL) associated with each radioactive peak were pooled and rechromatographed with the same system and gradient schedule, but buffer A was 0.1% trifluoroacetic acid and B was 0.07% trifluoroacetic acid in 75% acetonitrile. The final purification of the major labeled peptide was repeated with this system but with a different gradient: initial 98%; 5 min, 68%; 10 min, 68%; 50 min, 62%; 60 min, 20% with linear segments. Radioactive peptide fractions were analyzed for their amino acid content (Picotag System; Waters, Milford, MA) and sequence (Beckman System 890 spinning cup sequencer) as previously described (Bond & Strydom, 1989).

Expression and Purification of Recombinant χχ-ADH. χχ-ADH was expressed in *Escherichia coli* as a fusion protein with glutathione *S*-transferase (GST) from *Schistosoma japonicum* under the control of a *tac* promoter in the expression vector pGEX-2T (Pharmacia). The GST–χχ-ADH expression cassette, pL30-G4, was constructed by inserting the χχ-ADH cDNA into the pGEX-2T vector, as follows. Plasmid pGEX-2T was cut with *Bam*HI and *Eco*RI. χχ-ADH cDNA was liberated from clone 30L (Sharma et al., 1989) by *Nco*I/*Eco*RI digestion and subjected to three-fragment ligation with the cut expression vector and a duplex of oligonucleotides, Sta 1 and Sta 2 (Sta 1 = 5′-GAT CCC GCG TCA GCT TCC TGG GCC TGG ACC TGG ATG CGC AGC CCC GCT-3′; Sta 2 = 5′-CAT GGA GCG GGC CTG CGC ATC CAG GTC CAG GCC CAG GAA GCT GAC GCG G-3′). The resultant expression vector codes for a fusion protein with a spacer derived from the stalk sequence of human testis angiotensin-converting enzyme (Ehlers et al., 1989; our unpublished results) between the GST and ADH domains. This spacer facilitates interdomain cleavage by thrombin and leaves the rχχ-ADH enzyme with an 18 amino acid N-terminal extension. The mutation of Arg-115 to Ala by the two-step PCR overlap extension method of Ho et al. (1989) required the following four oligonucleotides (mutations underlined): E8-A = 5′-TGG AAA GTG TTG GTG AG-3′, E8-D = 5′-CCT CCC AGA CCA AAG AC-3′, E8-G = 5′-GCA GTC ACT CAA GGG AAA GGC CTA AT-3′, and E8-H = 5′-AGG CCT TTC CCT TGA GTG ACT GCT AT-3′. In the first stage, PCR amplifications with clone 30L as template were performed in parallel with E8-A and E8-H as primers in one reaction and E8-D and E8-G in the other. The two PCR products were purified by electrophoresis in a low-melt agarose gel, excised, and mixed to provide templates for the second stage of PCR amplification with E8-A and E8-D as primers. After phenol/chloroform extraction, the product of amplification was cleaved with *Dra*III and *Age*I (New England Biolabs, Beverly, MA) and purified by gel electrophoresis. The resulting *Dra*III/*Age*I fragment encodes the R115A mutation and contains a new *Stu*I restriction site. By replacing the corresponding fragment in pL30-G4 with this mutated *Dra*III/*Age*I segment, pL30-K4 was generated to serve as the expression plasmid for the R115A mutant. Sequences of all recombinant DNA fragments were confirmed by dideoxy sequencing of both strands (Sanger et al., 1977).

Proteins were expressed in the *E. coli* strain XL1-Blue (Stratagene, La Jolla, CA) by growing the cells in LB/ampicillin to an A_{600} of ~2 followed by induction with 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h. Cells were harvested and lysed, and the GST–χχ-ADH fusion protein was extracted with glutathione–agarose, as described (Smith & Johnson, 1988). The fusion protein bound to the affinity resin was washed three times with 10 mM Tris·HCl, pH 8.0, 2.5 mM $CaCl_2$, and 0.5 mM DTT and then cleaved by incubating the resin (50% suspension in washing buffer) with human plasma thrombin (Sigma, St. Louis, MO) at 37 °C for 2 h. After removal of solids by centrifugation, the cleavage product in the supernatant was applied directly to a DEAE 5PW anion-exchange HPLC column (Waters, Milford, MA) and chromatographed as described for the human enzyme (Moulis et al., 1991).

RESULTS

Arginine Modification. Phenylglyoxal, which reacts essentially irreversibly with arginine residues (Takahashi, 1968), eliminates the capacity of χχ-ADH to be activated by fatty acids (Figure 1A). Thus, incubation of χχ-ADH with 1 mM phenylglyoxal, pH 8.1, in 0.1 M sodium bicarbonate at ambient temperature results in a progressive increase, ~3-fold, in activity toward MCA over a 1-h period. However, the capacity of pentanoic acid to activate the enzyme is lost concurrently. MCA was used as substrate here since its 12-fold activation by pentanoate is conveniently determined. FDH activity of the enzyme (Figure 1B) is markedly diminished, as is that toward 12-HDA. After 40 min, when the residual FDH activity was 12%, that toward 12-HDA was 30%. The oxidation of this substrate, like that of HMGSH, is not subject to fatty acid activation. The changes induced by phenylglyoxal are irreversible since the enzyme, isolated after 1 h of incubation by gel filtration through a PD-10 column to remove

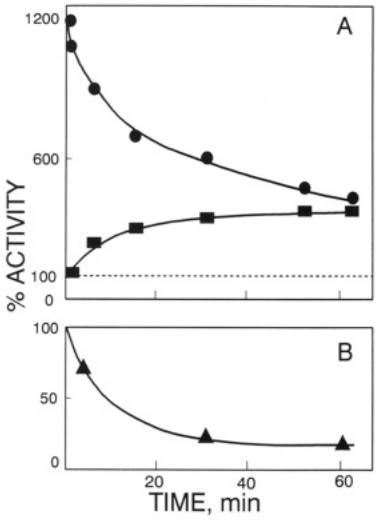

FIGURE 1: Changes in activity of χχ-ADH toward MCA and HMGSH on modification with phenylglyoxal. (A) Activity changes during the reaction monitored with MCA. At the times indicated aliquots were withdrawn and diluted 50-fold into the standard MCA assay (■). After the initial velocity was established, pentanoate was added to 50 mM to assess susceptibility to activation (●). (B) Activity toward HMGSH (▲).

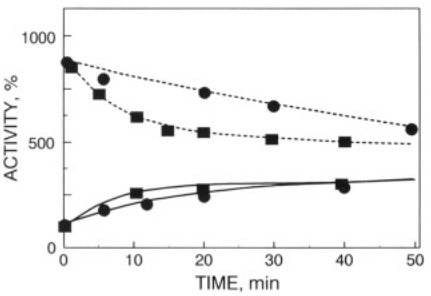

FIGURE 2: Protection by octanoate against modification of χχ-ADH with phenylglyoxal. Reactions were initiated by addition of phenylglyoxal to enzyme containing 6.25 mM octanoate (●) and 2.5 mM octanoate (■). At the times indicated aliquots were withdrawn and diluted 50-fold into the standard MCA assay (solid lines). After the initial velocity was established, pentanoate was added to 50 mM to assess susceptibility to activation (dashed lines).

reagents, remains insensitive to pentanoate and fails to recover FDH activity. A moderate increase in activity of ~2.8-fold, and a loss of fatty acid activation were also observed when 0.5 M ethanol was the substrate and 2 mM deoxycholate was the activator (not shown). The rate and extent of phenylglyoxal modification, assessed by monitoring MCA activity with and without added pentanoate are functions of the reagent concentration. Activation of only 2- and 1.4-fold is observed after 1 h of reaction with 0.2 and 0.5 mM phenylglyoxal, respectively, but treatment with 1 mM reagent essentially abolishes pentanoate activation. With 2 mM reagent loss of fatty acid activation is complete in 15 min. For convenience, 1 mM reagent was used in all further studies. The presence of 100 μM NAD^+ during modification does not protect against loss of fatty acid activation. However, octanoate, which induces half-maximal activation of ethanol oxidation at about 2 mM (Moulis et al., 1991), protects partially at 2.5 mM but much more so at 6 mM (Figure 2).

Phenylglyoxal Modification. Effects on Kinetic Parameters. The modification alters the activity toward dodecanol minimally (Table I) (both K_m and k_{cat} are changed by less than a factor of 2), but the k_{cat}/K_m for 12-HDA is decreased by almost 2 orders of magnitude, the result of a 15-fold increase in K_m and a 4-fold decrease in k_{cat}. The ω-carboxylate group

Table I: Kinetic Parameters for Dodecanol and 12-Hydroxydodecanoate Oxidation by Native and Phenylglyoxal-Modified χχ-ADH[a]

	native			Arg modified		
substrate	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
dodecanol	30	24	8×10^5	20	13	6.7×10^5
12-HDA	59	167	2.8×10^6	1000	39	3.9×10^4

[a] Units of K_m, k_{cat}, and k_{cat}/K_m are μM, min^{-1}, and M^{-1} min^{-1}, respectively. Assays were performed in 0.1 M glycine, pH 10.0, containing 2% methanol and 2.5 mM NAD^+.

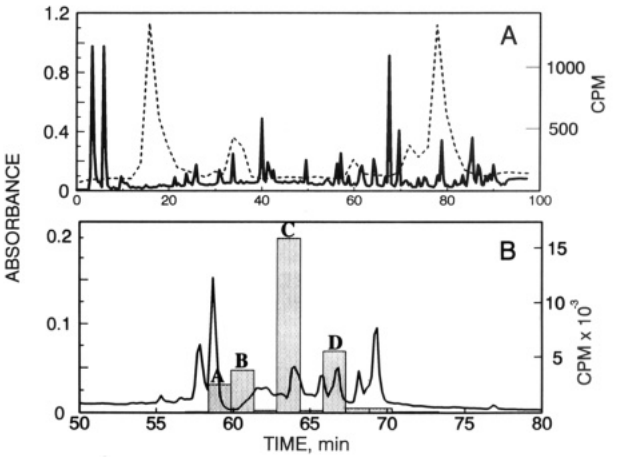

FIGURE 3: (A) HPLC isolation of [7-^{14}C]phenylglyoxal-labeled tryptic peptides following modification of χχ-ADH with 1 mM phenylglyoxal. Fractions of 1.5 mL were collected. Solid line: absorbance at 213 nm. Dashed line: radioactivity. (B) The pool from 75–84 min was rechromatographed under different conditions, and the fractions were monitored for absorbance and radioactivity as described in Materials and Methods. Solid line: absorbance at 213 nm. Bars: radioactivity.

apparently plays a substantial role in the catalytic process and could be a key factor in defining the specificity of χχ-ADH.

Stoichiometry of Phenylglyoxal Incorporation. Triplicate modifications of 1–2 mg of protein in 0.1 M bicarbonate, pH 8.1, with 1 mM phenylglyoxal established the stoichiometry of the reaction of [7-^{14}C]phenylglyoxal with χχ-ADH. After 1 h of reaction, the samples were washed extensively with bicarbonate buffer and radioactivity was measured. The average value was 3.5 mol of phenylglyoxal incorporated per 80 000 g of protein. Since 2 mol of phenylglyoxal combines with 1 mol of arginine (Takahashi, 1968) and the enzyme is a homodimer, this corresponds to 1 arginine residue modified per subunit.

Isolation of Phenylglyoxal-Labeled Peptides. χχ-ADH was reacted with phenylglyoxal as above, and the reaction was terminated after 20 min by gel filtration to limit excessive modification and diminish labeling heterogeneity (Means & Feeney, 1971). The product, containing 2.5 mol of phenylglyoxal per mol of χχ-ADH, was denatured, carboxymethylated, and digested with trypsin, and the peptides were mapped as described under Materials and Methods. To minimize potential loss of label, all manipulations from gel filtration to HPLC were carried out in a single Centricon filtration device.

The tryptic peptide map obtained by HPLC of the lyophilized sample shows two major regions of radioactivity, at about 19 and 80 min. (Figure 3A). These were collected, pooled, and analyzed for their radioactivity and amino acid content. The earlier major peak was essentially devoid of amino acids, in agreement with the nearly flat absorbance at 213 nm in this region, and was not analyzed further. The pool

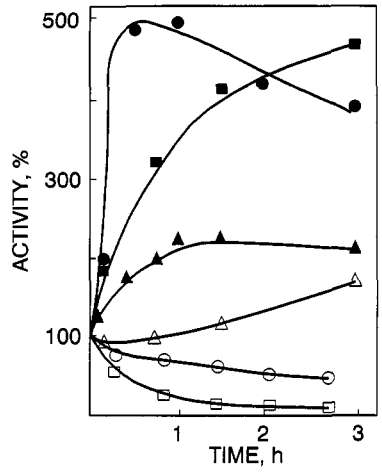

FIGURE 4: Reductive methylation of different alcohol dehydrogenases. The reactions were carried out with 2.5 μM enzyme, 1.5 mM formaldehyde, and 7.5 mM $NaBH_3CN$ in 50 mM Hepes, pH 7.5, as described in Materials and Methods, except for the horse enzyme, which was 2.75 μM and was reacted with 2 mM formaldehyde and 10 mM $NaBH_3CN$. □, χχ-ADH; ○, χχ-ADH in the presence of 100 mM NAD^+; ●, $\beta_1\beta_1$; ■, horse liver ADH; ▲, αα-ADH; △, horse liver ADH in the presence of 1 mM NAD^+.

from 75 to 84 min, containing 62% of the total radioactivity, was rechromatographed with trifluoroacetic rather than phosphoric acid containing buffers under slightly different conditions (Figure 3B). Fractions of 1.5 mL were collected, and four peaks (A–D) contained measurable radioactivity; over 56% of the total was in peak C. Each radioactive fraction was rechromatographed to give a single major band of absorbance and radioactivity, which was subjected to amino acid analysis.

The compositions of minor peaks A, B, and D were equivocal and did not fit clearly any peptide sequence of class III ADH. The major peak, C, had a composition of $S_{1.0}$-$G_{3.4}$-$T_{0.8}$-$A_{1.7}$-$Y_{0.2}$-$V_{0.9}$-$I_{0.7}$-$K_{0.8}$-$R_{0.4}$; no other amino acids were present above 0.1. This fraction produced an unequivocal sequence for six cycles of Edman degradation of I(79)-(X)-V(78)-T(20)-Q(57)-G(20) where the numbers in parentheses are the picomoles of amino acid recovered at each cycle from 113 pmol of peptide loaded. This uniquely fits the sequence from residues 114–119. Threonine at cycle 4 was verified by detection at 313 nm. The second cycle, indicated by the X, showed no clearly discernable product. From the sequence this residue should correspond to the modified arginine. This was verified by collecting the products at each cycle from the sequencer and examining them for radioactivity. All fractions showed background levels except cycle 2, which was 80 times above background. These data demonstrate that the labeled residue is Arg-115.

The yield of labeled peptide based on protein measurements of the gel-filtered protein was 5.3%, and that based on radioactivity was 2%. A loss of radioactivity up to 50% occurred at each HPLC step, the major one being in the initial tryptic digestion and subsequent chromatography, likely due to spontaneous loss of label from arginine (Takahashi, 1968) during the denaturation, carboxymethylation, and tryptic digestion procedures. Considering the multiple stages of purification, the yield is acceptable.

Reductive Methylation. Reductive methylation of χχ-ADH with formaldehyde/sodium cyanoborohydride almost completely abolishes activity toward ethanol in 3 h (Figure 4). In marked contrast, parallel experiments with the human $\beta_1\beta_1$ and αα isozymes as well as the horse EE enzyme all result in *increased* activity. The horse EE enzyme is known to be activated by lysine modification (Plapp, 1970; Jörnvall, 1973; Sogin & Plapp, 1975; Dubied et al., 1977), including reductive methylation (Tsai, 1983). It is activated 5-fold in 3 h, whereas the $\beta_1\beta_1$ enzyme reaches an equivalent activity after only 0.5 h, and αα is activated ~2-fold in 1 h. The presence of 1 μM NAD^+ during the reaction protects the horse enzyme from activation but does not affect the inactivation of χχ-ADH; even 100 mM NAD^+ affords only moderate protection. This clearly distinguishes χχ-ADH from the other enzymes examined with respect to response to methylation and suggests that lysine is not likely involved in activator binding.

Expression and Activity of the R115A Mutant. A recombinant expression system based on the glutathione transferase fusion protein served to produce low amounts of R115A, about 50 μg/L of culture, just sufficient for HPLC purification and initial examination of the effects of mutation. SDS–PAGE after HPLC indicates a dominant band of 42 kDa, slightly greater than the 40-kDa human liver class III ADH and in accord with the additional 18 amino acids at its N-terminus. Though minor bands are present, the major band of R115A represents about 80% of the total protein. Its specific activity toward 0.5 M ethanol, 0.36 unit/mg, is about one-half that of the human liver enzyme, 0.6 unit/mg (Moulis et al., 1991). Like the native enzyme, its K_m toward ethanol is above 3 M since initial velocities measured at 0.5 and 3 M ethanol are directly proportional to the ethanol concentration. Its specific activity toward HMGSH is 1.9 units/mg, compared to 3 units/mg for the native enzyme, and its apparent K_m, determined using two concentrations of HMGSH, 0.5 and 0.1 mM, is 300 μM, i.e., 75-fold higher than that of the native enzyme, 4 μM (Holmquist & Vallee, 1991). Addition of 50 mM pentanoate to the MCA assay to assess fatty acid activation produces a 2-fold increase in activity, compared to 12-fold for the native enzyme. Thus, mutation of Arg-115 to Ala has major effects on both substrate specificity and fatty acid activation.

DISCUSSION

Since the reaction stoichiometry of phenylglyoxal with arginine is 2:1 (Takahashi, 1968), the incorporation of 4 mol of [7-^{14}C]labeled reagent per mol of homodimeric enzyme indicates that a single arginine residue/subunit is modified. One major labeled peptide was isolated following denaturation, carboxymethylation, tryptic hydrolysis, and several stages of HPLC purification. The labeled peptide has an unambiguous sequence of Ile-X-Val-Thr-Gln-Gly consistent with the presence of a single major site of modification. The absence of an unidentifiable amino acid at cycle 2 in the Edman sequencing, which resulted in the only radioactive fraction, confirms that the sequence determined is in fact that of the labeled peptide. This sequence is unique to residues 114–119, where position 115 is arginine. Both flanking residues, 113 and 120, are lysines and potential sites of tryptic cleavage.

Labeling is restricted to one principal residue since fatty acid activation is lost completely with incorporation of only 1 "equivalent" of reagent per subunit of enzyme, and at that level of modification activity toward MCA reaches a plateau (Figure 1). The partial protection afforded by octanoate (Figure 2), an activator of ethanol activity with an apparent K_a of 2 mM (Moulis et al., 1991), further indicates selectivity of the reaction. Such selectivity is often seen with arginine modification (Riordan, 1979) and suggests either that Arg-115 is hyperreactive or that the other eight arginines/subunit are unreactive. Arg-115 is accessible to substrates and

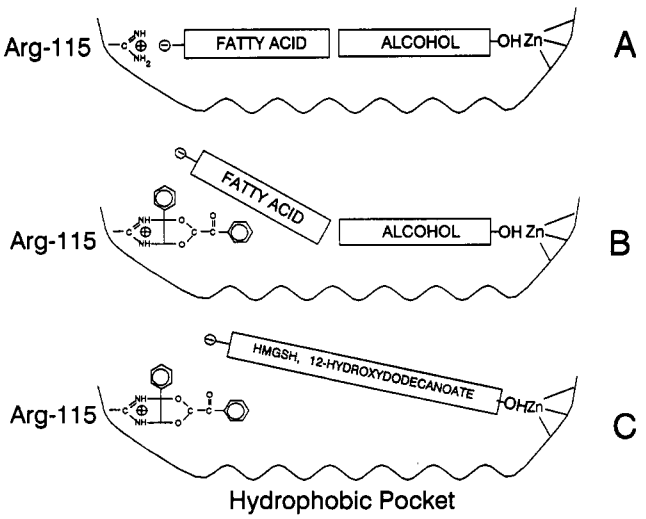

FIGURE 5: Diagram illustrating the proposed role of Arg-115 in the binding of activators and anionic substrates in the hydrophobic substrate binding pocket of χχ-ADH and the effects on activity upon modification of the enzyme by phenylglyoxal. The substrates are aligned such that their hydroxyl group is oriented for interaction with the active site zinc atom. Arg-115 is about 16 Å from the zinc and adjacent to Ile-116, a residue located in the substrate binding pocket. The binding of pentanoate to complement substrate binding (A) and activate the reaction with short-chain neutral alcohols is disrupted by the addition of the two phenylglyoxal molecules to Arg-115 (B); the activity toward the shorter alcohols is not diminished but is actually increased slightly. Binding to the modified enzyme by substrates that require Arg-115, presumably for electrostatic interaction, is curtailed with 12-HDA and HMGSH (C), resulting in a loss of activity.

activators and obviously also is free to react with phenylglyoxal. In contrast, the other arginines could be involved in interactions with other residues and, hence, unavailable to the reagent. Selectivity may also be enhanced by the relatively hydrophobic nature of the phenylglyoxal, which would direct it to the hydrophobic pocket.

The changes in activity of χχ-ADH on modification by phenylglyoxal are in accord with the proposed substrate and activator binding model (Figure 5). Complementary binding of a suitably sized fatty acid–substrate pair, such as pentanoate and MCA, would fully occupy the hydrophobic pocket and result in activation. In this case, activity is increased 12-fold, primarily due to a decrease in K_m. The addition of two bulky aromatic moieties to Arg-115 by phenylglyoxal modification apparently introduces sufficient hydrophobic character to mimic activator binding; the modified enzyme thus acts as an activator–enzyme complex. The 2- to 3-fold increased activity of the phenylglyoxal modified enzyme toward both ethanol and MCA may occur for this reason. However, the modification also blocks the interaction of Arg-115 with activators. As a consequence, activation by fatty acids is abolished. Either they no longer fit into the active site pocket or they cannot bind because a major component of their binding energy has been lost.

Blocking Arg-115 disrupts binding of, and activity toward, the longer substrates HMGSH and 12-HDA, but with neutral substrates such as ethanol and MCA the role of charge is much less detrimental. This is particularly evident from a comparison of the kinetic parameters of the native and modified enzymes toward dodecanol and 12-HDA. The kinetic parameters for dodecanol are essentially unchanged on modification, whereas those of 12-HDA reflect the loss of a significant binding component (Table I). The major effect is a 17-fold increase in K_m consistent with the role of the arginine as an anion binding site. The importance of a charge–charge interaction has also been seen with the ethyl ester of GSH, where esterification generates a 50-fold increase in K_m (Holmquist & Vallee, 1991).

Initial studies with the mutant R115A confirm both the identification of Arg-115 as the site of modification and its importance as a binding site for negatively charged activators and substrates, particularly HMGSH. R115A cannot be activated significantly by fatty acids and has extremely low FDH activity, and yet it has nearly the same specific activity toward ethanol as the native human liver enzyme. Further, modification of R115A by phenylglyoxal has no effect on either the MCA or the FDH activity of the enzyme (Engeland et al., 1993). More detailed studies of R115A and other mutants, prepared by an improved expression system, are reported elsewhere (Engeland et al., 1993).

The activation of χχ-ADH by arginine modification is in marked contrast to that of the human class I ADHs and the horse liver enzymes which are *inactivated* by both phenylglyoxal and butanedione (Lange et al., 1974; Jörnvall et al., 1977). In the horse enzyme, two arginine residues were modified, Arg-47 and Arg-84. The former participates in nucleotide binding (Eklund et al., 1991), and its modification was thought to be responsible for the inactivation. The latter is located on the surface of the enzyme opposite the catalytic domain and is not involved in activity. Neither of the corresponding positions of χχ-ADH is arginine, however. More importantly, residue 115 in the horse enzyme is not arginine but aspartic acid, while in none of the human isozymes of class I is position 115 an arginine. This accounts for the unique capacity of χχ-ADH to undergo activation by fatty acids and to exhibit FDH activity. Both the horse and the human class I isozymes are activated by lysine-specific agents such as methyl acetimidate and formaldehyde/borohydride, but this clearly differs from the inactivation observed here with χχ-ADH. In the horse enzyme the residue methylated is Lys-228, and the increased activity has been attributed to an increased rate of NADH dissociation. Reductive methylation of χχ-ADH (Figure 4) rapidly abolished activity despite the presence of Lys-228, indicating yet another unique property of this isozyme.

Where Is R115 and Why/How Is It Involved? An X-ray diffraction structure for χχ-ADH is not yet available, but extensive comparisons of its primary structure and computer-modeled tertiary structure with those of the horse EE enzyme (Eklund et al., 1990) have allowed functional assignment of a number of its residues. Arg-115 is among those that have not previously been invoked as being critical to any specific function. However, it is adjacent to Leu-116, one of the residues that lines the hydrophobic pocket of the horse enzyme. This pocket is thought to be responsible for the increased affinity for long hydrophobic substrates. Arg-115 is also about 16 Å from the catalytic zinc and is well positioned to assist in binding of ω-carboxy fatty acid substrates. A more detailed investigation of the kinetics and the role of Arg-115 in substrate and activator binding are presented in a separate paper (Engeland et al., 1993).

In summary, the reaction of χχ-ADH with the arginine-specific reagent phenylglyoxal results in the selective modification of a single arginine residue, Arg-115. The concomitant alterations in the specificity and fatty acid activation indicate a role for this residue in electrostatic association of negatively charged substrates or activators. The effects on activity (a slight activation by the reagent itself, loss of activation by fatty acids, and loss of FDH activity) indicate

that this residue is involved in substrate recognition but is not critical for catalytic function.

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REFERENCES

Beisswenger, T. B., Holmquist, B., & Vallee, B. L. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8369.

Bond, M. D., & Strydom, D. J. (1989) *Biochemistry 28*, 6110.

Dubied, A., von Wartburg, J. P., Bohlken, D. P., & Plapp, B. V. (1977) *J. Biol. Chem. 252*, 1464.

Ehlers, M. R. W., Fox, E. A., Strydom, D. J., & Riordan, J. F. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7741.

Eklund, H., & Brändén, C.-I. (1987) in *Biological Macromolecules and Assemblies* (Jurnak, F., & McPherson, A., Eds.) Vol. 3, pp 74–142, John Wiley & Sons, New York.

Eklund, H., Müller-Wille, P., Horjales, E., Futer, O., Holmquist, B., Vallee, B. L., Höög, J.-O., Kaiser, R., & Jörnvall, H. (1990) *Eur. J. Biochem. 193*, 303.

Engeland, K., Höög, J.-O., Holmquist, B., Estonius, M., Jörnvall, H., & Vallee, B. L. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 2491.

Gutheil, W. G., Holmquist, B., & Vallee, B. L. (1992) *Biochemistry 31*, 475.

Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., & Pease, L. R. (1989) *Gene 77*, 51.

Holmquist, B., & Vallee, B. L. (1991) *Biochem. Biophys. Res. Commun. 178*, 1371.

Jentoft, J. E., & Dearborn, D. G. (1981) *J. Biol. Chem. 254*, 4359.

Jörnvall, H. (1973) *Biochem. Biophys. Res. Commun. 51*, 1069.

Kaiser, R., Holmquist, B., Hempel, J., Vallee, B. L., & Jörnvall, H. (1988) *Biochemistry 27*, 1132.

Kaiser, R., Holmquist, B., Vallee, B. L., & Jörnvall, H. (1989) *Biochemistry 28*, 8432.

Koivusalo, M., Baumann, M., & Uotila, L. (1989) *FEBS Lett. 257*, 105.

Lange, L. G., Riordan, J. F., & Vallee, B. L. (1974) *Biochemistry 13*, 4361.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.

Maret, W., Anderson, I., Dietrich, H., Schneider-Bernlohr, H., Einarsson, R., & Zeppezauer, M. (1979) *Eur. J. Biochem. 98*, 501.

McEvily, A., Holmquist, B., & Vallee, B. L. (1990) *Biochromatography 5*, 13.

Means, G. E., & Feeney, R. E. (1971) in *Chemical Modification of Proteins*, pp 195–197, Holden-Day, San Francisco.

Moulis, J.-M., Holmquist, B., & Vallee, B. L. (1991) *Biochemistry 30*, 5743.

Parés, X., & Vallee, B. L. (1981) *Biochem. Biophys. Res. Commun. 98*, 122.

Plapp, B. V. (1970) *J. Biol. Chem. 245*, 1727.

Riordan, J. F. (1979) *Mol. Cell. Biochem. 26*, 71.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463.

Sharma, C. P., Fox, E. A., Holmquist, B., Jörnvall, H., & Vallee, B. L. (1989) *Biochem. Biophys. Res. Commun. 164*, 631.

Smith, D. B., & Johnson, K. S. (1988) *Gene 67*, 31.

Sogin, D. C., & Plapp, B. V. (1975) *J. Biol. Chem. 250*, 205.

Takahashi, K. (1968) *J. Biol. Chem. 243*, 6171.

Tsai, C. S. (1978) *Biochem J. 173*, 483.

Vallee, B. L., & Bazzone, T. J. (1983) *Curr. Top. Biol. Med. Res. 8*, 219.

Wagner, F. W., Burger, A. R., & Vallee, B. L. (1983) *Biochemistry 22*, 1857.

Wagner, F. W., Parés, X., Holmquist, B., & Vallee, B. L. (1984) *Biochemistry 23*, 2193.