Samuels, L. T., Helmrich, M. L., Lasuter, M. E., & Reinch, H. (1951) *Science 113*, 490–492.
Takahashi, M., Luu-The, V., & Labrie, F. (1990a) *Ann. N.Y. Acad. Sci. 595*, 383–385.
Takahashi, M., Luu-The, V., & Labrie, F. (1990b) *J. Steroid Biochem. Mol. Biol. 37*, 231–236.
Talalay, P., & Wang, V. S. (1955) *Biochim. Biophys. Acta 18*, 30–301.
Tashjian, J. A. H. (1979) in *Methods in Enzymology* (Jakoby, W. P., & Pastan, E., Eds.) Vol. 58, pp 527–535, Academic Press, New York.
Zhao, H. F., Rhéaume, E., Trudel, C., Couët, J., Labrie, F., & Simard, J. (1990) *Endocrinology 127*, 3237–3239.
Zhao, H. F., Labrie, C., Simard, J., Trudel, C., Martel, C., Rhéaume, E., Dupont, E., Luu-The, V., Pelletier, G., & Labrie, F. (1991) *J. Biol. Chem. 266*, 583–593.

Structural Organization of Aldehyde Dehydrogenases Probed by Limited Proteolysis†

Kerry Loomes‡ and Hans Jörnvall*
Department of Chemistry I, Karolinska Institutet, S-104 01 Stockholm, Sweden
Received February 19, 1991; Revised Manuscript Received May 29, 1991

ABSTRACT: Incubation of cytosolic and mitochondrial aldehyde dehydrogenases with trypsin or Glu-C protease under native conditions causes a time-dependent loss of dehydrogenase activity and the production of protein fragments. For evaluation of the results, termination of the reactions with a specific protease inhibitor is especially important in the case of the Glu-C protease. Cleavage site determination by SDS/polyacrylamide gel electrophoresis and sequence analysis identified protease-sensitive amino acid residues at two internal regions spanning positions 248–268 (region 1) and 397–399 (region 2) and at positions in the N-terminal segment (region 3). Region 1 encompasses several cleavages and is sensitive to both proteases in both aldehyde dehydrogenases. Further, it is in a conserved segment and correlates with reactive residues and regions ascribed functional roles. It also correlates with exon borders in the corresponding genes. Combined, the results define region 1 as an important and highly accessible segment of the protein. Region 2 is also adjacent to a conserved segment but lacks further correlation with special properties and appears just to represent an accessible region. The internally cleaved subunits retain a tetrameric configuration as calculated from exclusion chromatography and polyacrylamide gel electrophoresis under native conditions, suggesting that the quaternary structure is not dependent on covalently linked domains within the subunits. Furthermore, the fragments can bind to AMP–Sepharose, suggesting that some functional properties are retained within the cleaved tetramers. However, cleavage at position 35 appears to cause a large fragment (36–263) to be released from the tetramer, suggesting a role of an N-terminal segment or arm (at or before region 3) in subunit interactions.

Aldehyde dehydrogenase is an oxidoreductase with a wide variety of aldehyde substrates (Blackwell et al., 1989). The NAD^+-dependent cytosolic and mitochondrial mammalian enzymes are homotetramers with known primary structures, analyzed at the protein level for the two forms from both human (Hempel et al., 1984, 1985) and horse (von Bahr-Lindström et al., 1984; Johansson et al., 1988) liver. Corresponding cDNA and genomic structures have been analyzed for these and other mammalian forms of the two types of enzyme (Hsu et al., 1985, 1988, 1989; Braun et al., 1987; Dunn et al., 1989; Farrês et al., 1989), as well as for prokaryotic aldehyde dehydrogenases from three species (Pickett et al., 1987; O'Connell & Kelly, 1989; Kok et al., 1989). Furthermore, another type of aldehyde dehydrogenase, dimeric and active also with $NADP^+$, has been structurally characterized from rat hepatocarcinoma and from liver after induction with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Jones et al., 1988; Hempel et al., 1989). It appears identical with or highly similar to forms from normal bladder, stomach, and other organs (Lindahl, 1986; Algar & Holmes, 1989; Eckey et al., 1990; Yin et al., 1991). While the two tetrameric mammalian enzyme types exhibit 68% residue identity, the dimeric type is clearly different, representing another class with a residue identity at only about the 30% level. Consequently, at least three mammalian forms of aldehyde dehydrogenase exist with largely known interrelationships.

Structural comparisons of the tetrameric enzymes gave an initial estimate of the organization of the molecules, localizing likely segments for coenzyme binding, and active-site residues (Hempel et al., 1985), as later also concluded from knowledge of the dimeric enzyme (Hempel et al., 1989). In spite of this structural knowledge, there is little information available on the tertiary structure and only limited insight into the functional organization of the enzyme molecule. Important roles have been ascribed to Lys-487 from natural variants of the mitochondrial enzyme (Yoshida et al., 1984), and to positions

† Supported by the Swedish Medical Research Council (Project O3X-3532) and the Wenner-Gren Foundation, Stockholm, Sweden (fellowship to K.L.).
* Address correspondence to this author.
‡ Present address: Department of Biochemistry, University of Auckland, Private Bag, Auckland, New Zealand.

0006-2960/91/0430-8865$02.50/0

Ser-74, Glu-268, and Cys-302 from chemical modifications with substrate analogues, coenzyme analogues, or other reagents (Hempel et al., 1982a; von Bahr-Lindström et al., 1985; Abriola et al., 1987; Loomes et al., 1990; Blatter et al., 1990). Additional positions have also been ascribed special functional significance. However, of the groups implicated at the active site, only Glu-268 and Cys-302 (using the nomenclature of the human/horse cytosolic and mitochondrial enzymes) are conserved in all 11 mammalian and prokaryotic primary structures known to date.

In the present study, cytosolic and mitochondrial aldehyde dehydrogenases from human liver are subjected to limited enzymatic proteolysis in order to probe the tertiary structure by identification of regions sensitive to proteolysis, and to correlate segments with specific functions. Interestingly, regions sensitive to limited proteolysis are found and define an important internal segment as well as a possible N-terminal "arm" involved in subunit interactions.

Materials and Methods

Aldehyde Dehydrogenase. Mitochondrial and cytosolic aldehyde dehydrogenases from human liver were purified as described (Hempel et al., 1982b) and stored at 4 °C in 50 mM sodium phosphate, pH 7.8, containing EDTA (1 mM) and dithiothreitol (30 mg/mL). Enzyme assays were performed at 25 °C using a Beckman DU-68 spectrophotometer to monitor the increase of NADH as measured at 340 nm in 0.1 M sodium pyrophosphate, pH 9.0. Protein concentrations were calculated by using extinction coefficients at 280 nm of 0.96 for the cytosolic enzyme and 1.00 for the mitochondrial enzyme (Greenfield & Pietruszko, 1977).

Limited Proteolysis. Digestions were performed by incubation of cytosolic and mitochondrial aldehyde dehydrogenases (1.8 mg/mL) with TPCK-trypsin (Worthington) (protease: substrate ratio of 1:30, w/w) or Glu-C protease (Boehringer) (protease:substrate ratio 1:5, w/w) at 37 °C in 50 mM sodium phosphate, pH 7.8, containing EDTA (1 mM) and dithiothreitol (30 mg/mL). In some experiments, proteolysis was performed in 0.1 M ammonium bicarbonate/EDTA (1 mM), pH 8.0, or 0.1 M Tris-HCl/EDTA (1 mM), pH 8.0. At specified times, aliquots were removed from the digestion mixtures and assayed for dehydrogenase activity. In addition, proteolysis activity was inhibited by the addition of phenylmethanesulfonyl fluoride to 1 mM final concentration for the tryptic digest, and (3,4-dichlorophenyl)isocoumarin to 200 μM final concentration for the Glu-C digest, and incubation for 2 min at 37 °C. The samples were then added to SDS/polyacrylamide gel electrophoresis solubilization buffer (70 mM Tris-HCl, pH 6.8, 2.5% SDS, 0.02% bromophenol blue, and 5% 2-mercaptoethanol), heated for 5 min at 110 °C, and subjected to SDS/polyacrylamide gel electrophoresis.

Enzyme Activity. Esterase activity with 100 μM 4-nitrophenyl acetate was assayed at 25 °C in 0.1 M pyrophosphate, pH 9.0, by measurement of the increase in the 4-nitrophenoxide ion as monitored at 400 nm. Values were corrected for the spontaneous hydrolysis of 4-nitrophenyl acetate. Dehydrogenase activity was measured as described above, and is, unless otherwise stated, the activity referred to in the text.

Fragment Purification. For preparative experiments, aldehyde dehydrogenase was incubated at 37 °C with TPCK-trypsin or Glu-C protease in 0.1 M ammonium bicarbonate, pH 8.0, and EDTA (1 mM). The digests were subjected to slab gel SDS/polyacrylamide gel electrophoresis, electroblotted (Bergman & Jörnvall, 1987), and analyzed for N-terminal sequence with a 470A Applied Biosystems sequencer with reverse-phase HPLC (Hewlett Packard 1090) phenylthiohydantoin identification (Kaiser et al., 1988).

In addition to separation by SDS/polyacrylamide gel electrophoresis, fragments were isolated by separation of incubation mixtures containing aldehyde dehydrogenase (3 mg) and the protease on a Superose-12 column equilibrated with 50 mM sodium phosphate, pH 7.8, EDTA (1 mM), and dithiothreitol (30 mg/mL).

Binding Experiments. A digestion mixture containing mitochondrial aldehyde dehydrogenase (1.4 mg) and trypsin (protease:enzyme ratio 1:30, w/w) in 0.1 M ammonium bicarbonate, pH 8.0, was incubated for 10 min at 37 °C and loaded onto a Mono Q anion-exchange column at 4 °C, equilibrated with 5 mM sodium phosphate, pH 6.8. Native, undigested enzyme and fragments were eluted in the same fraction by application of a linear gradient to 100 mM sodium phosphate, pH 6.8. The fraction which contained both intact and cleaved enzyme was loaded onto an AMP–Sepharose column (12 mm × 180 mm) equilibrated with 30 mM sodium phosphate, pH 6.0. After the column was washed with this buffer, bound protein and fragments were eluted by the addition of NADH (0.5 mg/mL).

Molecular Weight Standards. Protein fragments from SDS/polyacrylamide gel electrophoresis, polyacrylamide gel electrophoresis, and exclusion chromatography were evaluated by using calibration curves from measurements with the commercially available standard proteins ribonuclease A, chymotrypsinogen A, ovalbumin, bovine serum albumin, aldolase, catalase, ferritin, and thyroglobulin.

Results

Activity Loss. The effects of four different proteases, trypsin, Glu-C protease, Lys-C protease, and chymotrypsin, were tested by activity measurements and by SDS/polyacrylamide gel electrophoresis. All appeared to give limited cleavages, most clearly with trypsin and the Glu-C protease. Consequently, these two proteases were used in further analyses. Their effects on the dehydrogenase activity of cytosolic and mitochondrial aldehyde dehydrogenases are shown in Figure 1A. In all cases, the incubation results in a reproducible, time-dependent decrease in activity, with trypsin being the more effective of the two proteases in bringing about inhibition. These profiles were reproducibly obtained regardless of the buffer system used (sodium phosphate, ammonium bicarbonate, Tris-HCl; cf. Materials and Methods). Controls containing aldehyde dehydogenases without protease were stable under the reaction conditions. As obvious from Figure 1B, the dehydrogenase and esterase activities appear to fall in parallel.

The effects of coenzyme and substrate (propionaldehyde) on the loss of activity upon protease treatment are shown in Figure 1C for the mitochondrial enzyme and trypsin. With either 500 μM NAD^+, 500 μM NADH, or 20 mM propionaldehyde (concentrations sufficient to saturate the enzyme; Feldman & Weiner, 1972; Ambroziak et al., 1989) present during the protease incubation, there was little effect on the rate of activity loss. However, when *both* NADH and propionaldehyde were present, a substantial protection against activity loss was observed; SDS/polyacrylamide gel electrophoresis showed that this protection was directly attributable to a slower rate of enzyme digestion. As propionaldehyde had little effect alone, the protection appears not to be attributed simply to direct Schiff base protein modification with the aldehyde. Similarly, the finding that coenzyme alone was ineffective suggests that conformational factors, brought about by coenzyme binding, are not the sole explanation of the protection. Consequently, factors influencing the activity may

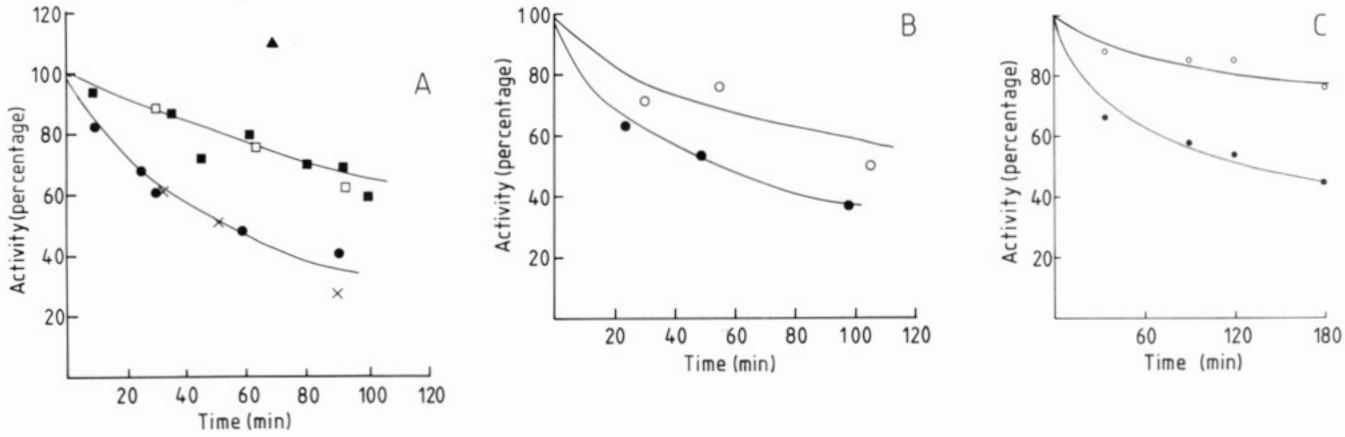

FIGURE 1: Inactivation profiles for aldehyde dehydrogenase by trypsin and Glu-C protease: (A) dehydrogenase activity followed with time; (B) comparison of the esterase and dehydrogenase activities; (C) protective effects of NADH and substrate on the dehydrogenase activity. (A) Incubation mixtures containing cytosolic aldehyde dehydrogenase and trypsin (1:30 w/w) (●), or Glu-C protease (1:5 w/w) (×), mitochondrial aldehyde dehydrogenase, and trypsin (1:30 w/w) (□), or Glu-C protease (1:5 w/w) (■) were incubated in 50 mM sodium phosphate, pH 7.8, EDTA (1mM), and dithiothreitol (30 mg/mL) at 37 °C. Experimental values for each digest are from at least two identical experiments. (▲) shows a control with mitochondrial aldehyde dehydrogenase incubated under identical conditions without protease. (B) Cytosolic aldehyde dehydrogenase and trypsin (1:30 w/w) under the same conditions as in (A) showing the dehydrogenase activity (○) and the esterase activity with 4-nitrophenyl acetate (●). (C) Incubation mixtures containing mitochondrial enzyme and trypsin (1:30 w/w) at 37 °C in 0.1 M Tris-HCl, pH 8.0, in the absence (●) and presence (○) of 500 μM NADH and 20 mM propionaldehyde.

be more specific or combined.

Fragment Generation and Identification. In early experiments, generation of fragments was evaluated by SDS/polyacrylamide gel electrophoresis, relying on SDS and heat denaturation for termination of the protease action during the preelectrophoresis boiling in the application buffer. However, these results were sometimes irreproducible, without proper correlation between patterns of enzyme inhibition and fragment generation, as evaluated by the electrophoresis results. It was apparent that in the absence of specific protease inhibitors, particularly with the Glu-C protease, cleavage sometimes occurred after the addition of solubilization buffer and resulted in further digestion of protein. Therefore, (3,4-dichlorophenyl)isocoumarin was added to the digests with Glu-C protease to ensure protease inhibition before evaluation of fragment patterns (Harper et al., 1985). Similar problems with the tryptic digestion mixtures were not found, but phenylmethanesulfonyl fluoride was nevertheless added to these digests before addition of the preelectrophoresis denaturation buffer. We also found that Glu-C protease and trypsin could be removed from the digestion mixtures (Figure 4, below) before SDS/polyacrylamide gel electrophoresis.

For both the cytosolic and the mitochondrial dehydrogenases, inhibition by trypsin or Glu-C protease is associated with the production of specific protein fragments, as indicated by SDS/polyacrylamide gel electrophoresis (Figure 2). Fragment patterns were the same regardless of whether SDS/polyacrylamide gel electrophoresis was carried out in the presence or absence of 2-mercaptoethanol. In all cases, the extent of removal of intact subunits roughly correlates with the loss of activity. The molecular masses of the fragments were estimated from SDS/polyacrylamide gel electrophoresis and correlated with the cleavage identifications obtained from sequence analysis (Figure 3).

For the mitochondrial enzyme and trypsin, fragments were found with molecular masses of approximately 29, 26, and 25 kDa (Figure 2A). Their cleavage points were determined by electroblotting and sequence analysis to be as shown in Figure 3. For the 26-kDa fragment, a minor cleavage was identified at Arg-264, in addition to the major one at Lys-263 in Figure 3, while for the 25-kDa fragment a minor cleavage was detected at Lys-35. N-Termini were identified from Ala-7 and

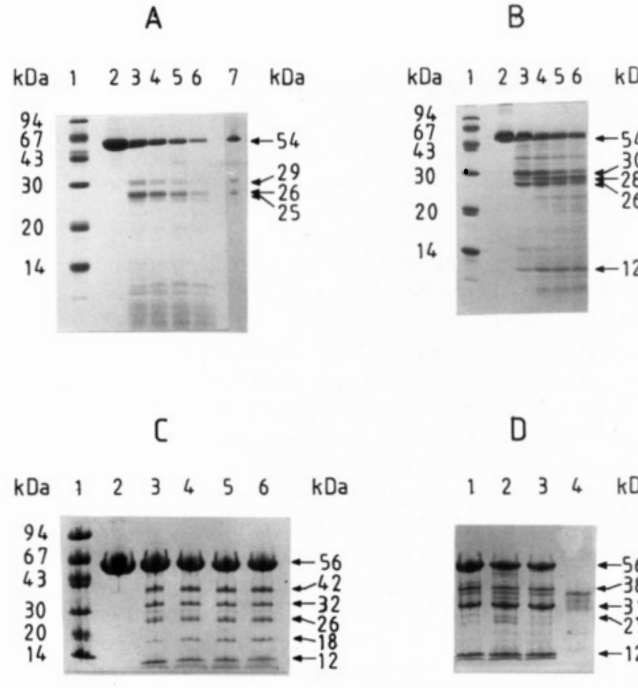

FIGURE 2: SDS/polyacrylamide gel electrophoresis of aldehyde dehydrogenase fragments after limited digestions with trypsin and Glu-C protease. (A) Mitochondrial aldehyde dehydrogenase and trypsin; (B) mitochondrial aldehyde dehydrogenase and Glu-C protease; (C) cytosolic aldehyde dehydrogenase and trypsin; (D) cytosolic aldehyde dehydrogenase and Glu-C protease. Protease:protein ratios were 1:30 (w/w) in (A) and (C) and 1:5 in (B) and (D). Incubations at 37 °C in 0.1 M ammonium bicarbonate, pH 8, in (A) and (B) and in 50 mM sodium phosphate, pH 7.8, EDTA (1 mM), and dithiothreitol (30 mg/mL) in (C) and (D). Analyses are shown at different extents of inhibition versus the dehydrogenase activity at zero incubation time. (A) Standard markers (lane 1); mitochondrial aldehyde dehydrogenase, no protease (lane 2); at 40% (lane 3), 69% (lane 4), 80% (lane 5), and 90% (lane 6) inhibition; and the 29-, 26-, and 25-kDa bands at higher resolution (lane 7). (B) Standard markers (lane 1); mitochondrial aldehyde dehydrogenase, no protease (lane 2); and at 10% (lane 3), 20% (lane 4), 35% (lane 5), and 58% (lane 6) inhibition; (3,4-dichlorophenyl)isocoumarin was not added prior to boiling. (C) Standard markers (lane 1); cytosolic aldehyde dehydrogenase, no protease (lane 2); and at 22% (lane 3), 40% (lane 4), 60% (lane 5), and 72% (lane 6) inhibition. (D) Cytosolic aldehyde dehydrogenase at 50% (lane 1), 70% (lane 2), and 75% (lane 3) inhibition; commercial Glu-C protease (lane 4).

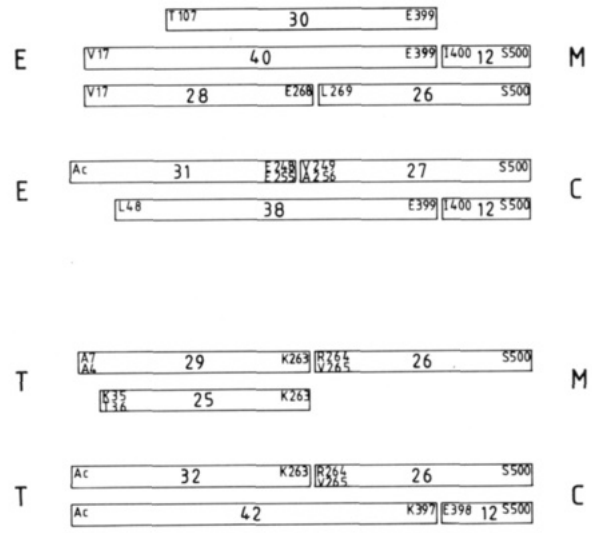

FIGURE 3: Fragment assignments for the Glu-C-derived (E) and tryptic-derived (T) cleavages of mitochondrial (M) and cytosolic (C) aldehyde dehydrogenases. The N-termini of the nonblocked fragments were identified by sequence analysis, while the C-terminal and N-acetylated assignments were deduced from estimated fragment molecular mass from SDS/polyacrylamide gel electrophoresis and by comparison with the known primary structures. Numbers indicate the estimated fragment molecular masses in kilodaltons, while terminal amino acid residues are denoted by one-letter abbreviations and positional numbers. In the case with trypsin, the 25- and 26-kDa fragments showed two adjacent start points because of cleavages at dibasic residue sequences. For the 12-kDa fragments, analyses were determined only with the cytosolic aldehyde dehydrogenase. Sequence analyses of the fragments of the cytosolic enzyme were performed after gel filtration on Superose-12.

Ala-4, probably indicating an N-terminal heterogeneity of the mitochondrial enzyme as has been found previously (Hempel et al., 1985).

For mitochondrial aldehyde dehydrogenase and Glu-C protease, major fragments appear at positions corresponding to 30, 28, 26, and 12 kDa, but are susceptible to further degradation as indicated by the complicated gel pattern in Figure 2B. Consequently, the fragments from the Glu-C digests are more labile than those from the tryptic digestion. The major cleavages with Glu-C protease, as found from sequence analysis, are shown in Figure 3.

For the cytosolic enzyme and either trypsin or Glu-C protease, the SDS/polyacrylamide gel electrophoresis patterns are shown in Figure 2C,D, while the cleavage assignments are shown in Figure 3. For the cytosolic enzyme and trypsin, the 26-kDa fragment (Figure 3) showed a minor cleavage at Arg-264.

Quaternary Structure. To investigate the quaternary structure of the proteolytically cleaved aldehyde dehydrogenases, the digests of the cytosolic and mitochondrial enzymes were subjected to exclusion chromatography, as shown in Figure 4A. For each digest, SDS/polyacrylamide gel electrophoresis of the material corresponding to the major peak showed fragments and intact aldehyde dehydrogenase, suggesting that they coelute (Figure 4B). Furthermore, the major peaks appeared to be well separated from both Glu-C protease and trypsin, as shown in Figure 4A.

For the tryptic-derived fragments of the mitochondrial enzyme, however, a somewhat different pattern was noticed. Thus, while the 29- and 26-kDa fragments were retained (lane 7, Figure 4B) in the fraction eluting in the major peak, the 25-kDa species was partially lost from this material and appeared later in the profile from the exclusion chromatography (Figure 4A and lane 8, Figure 4B). This was confirmed by

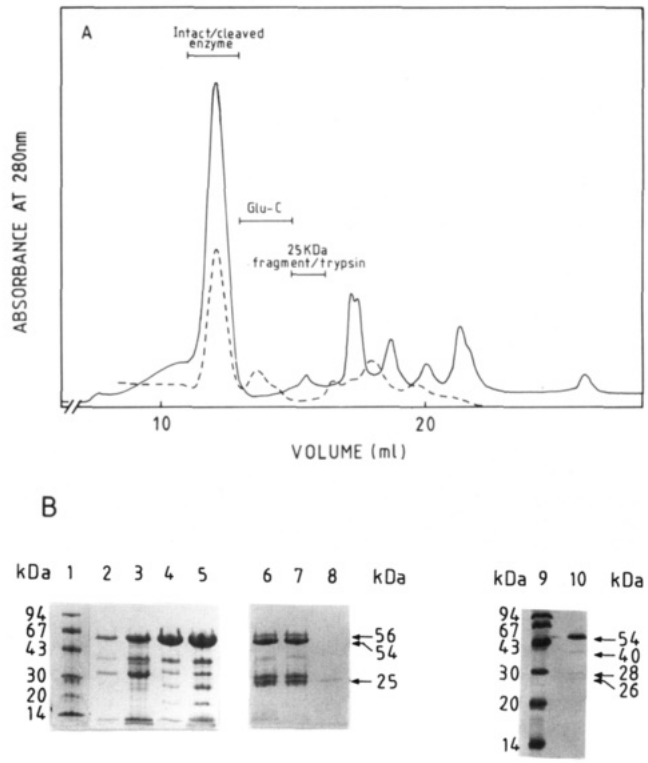

FIGURE 4: Comparisons of gel filtration (A) and SDS/polyacrylamide gel electrophoresis (B) of aldehyde dehydrogenase fragments after proteolysis. (A) After incubation in 50 mM sodium phosphate, pH 7.8 (containing EDTA and dithiothreitol), the digest was loaded onto a Superose-12 column equilibrated with the same buffer. The profiles for cytosolic aldehyde dehydrogenase/trypsin and mitochondrial aldehyde dehydrogenase/trypsin were virtually identical (solid line), as were the profiles for the cytosolic aldehyde dehydrogenase/Glu-C protease and mitochondrial aldehyde dehydrogenase/Glu-C protease (dashed line). (B) SDS/polyacrylamide gel electrophoresis of tryptic and Glu-C digests of aldehyde dehydrogenase and of the material corresponding to the major peaks from gel filtration. Lanes 2–5 represent analyses for the cytosolic enzyme; lanes 6–10 for the mitochondrial enzyme. Lane 1, standards; lane 2, Glu-C-derived fragments from the main peak (75% inhibition of dehydrogenase activity before electrophoresis; lane 3, Glu-C digest before being loaded (36% inhibition); lane 4, tryptic-derived fragments from the main peak (35%); lane 5, tryptic digest before being loaded (68%); lane 6, tryptic digest before being loaded (50%); lane 7, tryptic-derived fragments from the main peak (50%); lane 8, the 25-kDa fragment; lane 9, standards; lane 10, Glu-C-derived fragments from the major peak (33%). Lanes 6 and 7 show some contamination with the cytosolic enzyme at 56 kDa.

sequence analysis of the material corresponding to the major peak containing the 29- and 26-kDa fragments, which revealed only two sequences, one starting from Ala-7 and the other from Val-265. The 25-kDa species, starting at Thr-36, was shown by sequence analysis to be derived from the material eluting as shown in Figure 4A. The fraction containing the 25-kDa fragment exhibited no dehydrogenase activity, no esterase activity with 4-nitrophenyl acetate, and no fluorescence interaction with NADH.

The material corresponding to the major peaks from Figure 4A for both cytosolic and mitochondrial enzyme digestions was rechromatographed on Superose-6 under native conditions. In all cases, the elution positions of peak fractions from gel filtration were identical with controls containing native, tetrameric aldehyde dehydrogenase, at approximately 213 kDa. There was no evidence for dimers or other species arising from tetramer dissociation. Polyacrylamide gel electrophoresis under native conditions using both Coomassie and activity staining confirmed this. Attempts to isolate the fragments by dissociation with 5 M urea were unsuccessful. Consequently,

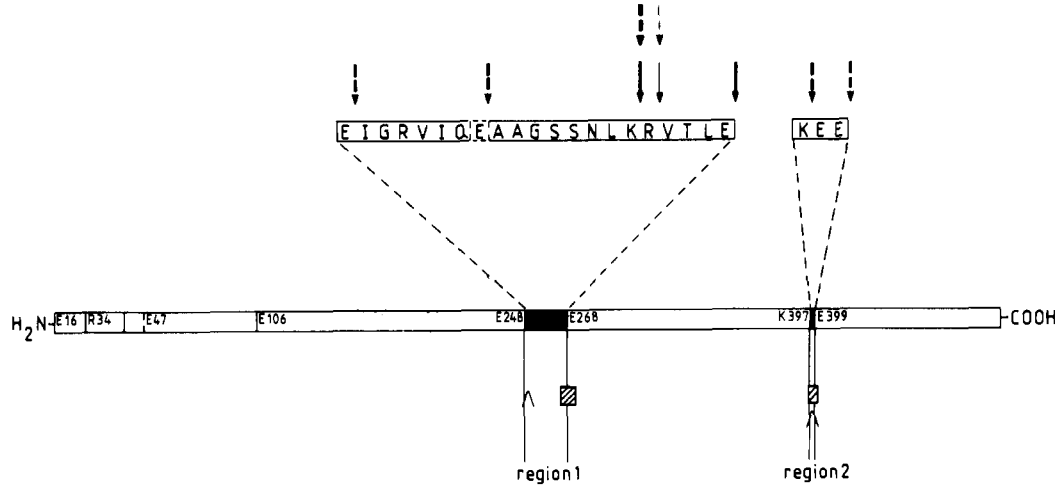

FIGURE 5: Schematic representation of the cleavages identified from limited proteolysis of both cytosolic and mitochondrial aldehyde dehydrogenases. Major and minor cleavages are denoted by bold and light arrows, respectively, and regions 1 and 2 are shaded as shown. Dashed lines show cleavage points for the cytosolic enzyme and solid lines for the mitochondrial enzyme. Known exon boundaries for the mitochondrial enzyme (Hsu et al., 1988) in the vicinities of regions 1 and 2 are shown with arrows below the primary structure. The hatched boxes denote two segments of conserved primary structure in all primary structures investigated.

these findings suggest that the cleaved aldehyde dehydrogenases retain a tetrameric configuration.

The digest containing mitochondrial aldehyde dehydrogenase and trypsin was purified on a Mono Q DEAE-anion exchanger followed by an AMP–Sepharose step. SDS/polyacrylamide gel electrophoresis of the material eluted from AMP–Sepharose with NADH showed intact enzyme and the 29- and 26-kDa fragments as the major constituents, demonstrating that the enzyme with some cleaved subunits in the tetramer can still bind coenzyme.

Discussion

Structurally Important Regions. For both cytosolic and mitochondrial aldehyde dehydrogenases, a pattern emerges which reveals a few regions especially sensitive to proteolysis under native conditions. They are near the N-terminus, and at two internally positioned specific areas in the primary structure (regions 1 and 2, Figure 5). The latter areas span segments containing residues 248–268 and 397–399 and are near positions corresponding to exon boundaries in the gene. Furthermore, both regions 1 and 2 overlap two segments of primary structure from 265–274 and 398–403, respectively, which are invariant in the primary structures from tetrameric mammalian aldehyde dehydrogenases (Hempel et al., 1984, 1985; von Bahr-Lindström et al., 1984; Johansson et al., 1988), three prokaryotic aldehyde dehydrogenases (Pickett et al., 1987; Kok et al., 1989; O'Connell & Kelly, 1989), and a characterized dimeric mammalian aldehyde dehydrogenase (Jones et al., 1988; Hempel et al., 1989). The invariance, together with the finding that trypsin and Glu-C protease independently cleave these regions for both the cytosolic and mitochondrial aldehyde dehydrogenases, strongly suggests regions 1 and 2 to be important and accessible elements in the basic structure.

Previous studies have suggested that the region containing Gly-245 and Gly-250 may play a role in coenzyme binding (Hempel et al., 1984, 1985). This segment is positioned at the N-terminal side of region 1. On the other hand, Cys-302 has been strongly implicated to be a residue of direct functional importance, possibly catalytic (Hempel et al., 1982a; von Bahr-Lindström et al., 1985; Blatter et al., 1990), and is situated further on the C-terminal side. Consequently, the data are consistent with coenzyme binding and catalytic sites being positioned on the N- and C-terminal sides of region 1, respectively. Furthermore, both regions 1 and 2 coincide with exon boundaries (Figure 5) in the genes (Hsu et al., 1988, 1989), and this is also compatible with the suggestion that region 1 connects two functional parts of the enzyme. The conserved Val^{265}-Pro^{274} segment which is adjacent to region 1 contains Glu-268, a residue suggested to reside at the active site (Abriola et al., 1987). An attractive possibility compatible with the data is that it could orient the coenzyme and catalytic parts in such a way that the conserved segment is positioned at the active site. Here, NADH and propionaldehyde combined (see Results) may protect against activity loss by forming a tertiary dead-end complex which slows the rate of cleavage at region 1.

Recently, a positional similarity between the cysteine protease family and the C-terminal parts of aldehyde dehydrogenases has been suggested (Hempel et al., 1991). This appears to start from position 284 of mammalian dehydrogenases. This is consistent with the present findings and suggests that catalytic parts of aldehyde dehydrogenase may correspond to those of cysteine protease functional units.

Region Important for the Quaternary Structure. Exclusion chromatography and polyacrylamide gel electrophoresis under native conditions show that the fragments derived from cytosolic and mitochondrial aldehyde dehydrogenases retain a tetrameric configuration. Furthermore, in the case of the mitochondrial enzyme and trypsin, the cleaved enzyme can still bind to AMP–Sepharose. Thus, most of the cleavages do not affect intersubunit binding segments, and the fragments cleaved are retained within the tetramer. The stoichiometry of cleaved to uncleaved subunits within the tetramer is difficult to judge as both intact and cleaved enzymes are present in the proteolysis mixtures, but the correlation between activity loss and extent of subunit digestion suggests that all subunits in the tetramer are amenable to proteolysis.

For mitochondrial aldehyde dehydrogenase and trypsin, the 29-kDa (starting at the N-terminus) and 26-kDa (starting at Val-265) fragments (Figure 4B) are retained in the tetramer, but under identical conditions, the 25-kDa fragment (starting at Thr-36) appears to be preferentially lost, and to a large extent recovered at a separate position eluting later during exclusion chromatography. This implies that the 35-residue N-terminal arm, which is missing in the 25-kDa species, is important in subunit interactions and may be a major factor contributing to the retention of the 29-kDa fragment. This

pattern with an N-terminal arm of importance in tetramer formation resembles one property of the known relationships for lactate dehydrogenase. Thus, the subunits of this enzyme possess an N-terminal, projecting arm contributing to tetramer stability, and loss of the arm can cause dissociation into dimers (Holbrook et al., 1975). For both cytosolic and mitochondrial aldehyde dehydrogenases, a similar arrangement with a projecting subunit-binding "arm" is consistent with the observed fragment patterns and their effects on the protein. Furthermore, it may be noticed that dimeric aldehyde dehydrogenase lacks the 56-residue N-terminal segment of the cytosolic and mitochondrial aldehyde dehydrogenases (Hempel et al., 1989). Thus, both natural variants of aldehyde dehydrogenases and the present demonstration of dissociation upon one type of limited proteolysis implicate the involvement of an N-terminal arm in stabilization of the tetramer.

ACKNOWLEDGMENTS

We are indebted to Drs. Maria Krook and Patrick Rouimi for useful discussions and to Carina Palmberg for help with the figures.

Registry No. aldehyde dehydrogenase, 9028-86-8.

REFERENCES

Abriola, D. P., Fields, R., Stein, S., MacKerell, A. D., Jr., & Pietruszko, R. (1987) *Biochemistry 26*, 5679–5684.

Algar, E. M., & Holmes, R. S. (1989) *Biochim. Biophys. Acta 995*, 168–173.

Ambroziak, W., Kosley, L., & Pietruszko, R. (1989) *Biochemistry 28*, 5367–5373.

Bergman, T., & Jörnvall, H. (1987) *Eur. J. Biochem. 169*, 9–12.

Blackwell, L. F., MacGibbon, A. K. H., & Buckley, P. D. (1989) in *Human Metabolism of Alcohol* (Crow, C. E., & Batt, R. D., Eds.) Vol. 2, pp 89–104, CRC Press, Boca Raton, FL.

Blatter, E. E., Tasayco, M. L., Prestwich, G., & Pietruszko, R. (1990) *Biochem. J. 272*, 351–358.

Braun, T., Bober, E., Singh, S., Agarwal, D. P., & Goedde, H. W. (1987) *FEBS Lett. 215*, 233–236.

Dunn, T. J., Koleske, A. J., Lindahl, R., & Pitot, H. C. (1989) *J. Biol. Chem. 264*, 13057–13065.

Eckey, R., Agarwal, D. P., Hempel, J., Timman, R., & Geodde, H. (1990) *Abstr. Enzymol. Mol. Biol. Carbonyl Metab.*

Farrês, J., Guan, K.-L., & Weiner, H. (1989) *Eur. J. Biochem. 180*, 67–74.

Feldman, R., & Weiner, H. (1972) *J. Biol. Chem. 247*, 267–272.

Greenfield, N. J., & Pietruszko, R. (1977) *Biochim. Biophys. Acta 483*, 35–45.

Harper, J. W., Hemmi, K., & Powers, J. C. (1985) *Biochemistry 24*, 1831–1841.

Hempel, J., Pietruszko, R., Fietzek, P., & Jörnvall, H. (1982a) *Biochemistry 21*, 6834–6838.

Hempel, J. D., Reed, D. M., & Pietruszko, R. (1982b) *Alcohol.: Clin. Exp. Res. 6*, 417–425.

Hempel, J., von Bahr-Lindström, H., & Jörnvall, H. (1984) *Eur. J. Biochem. 141*, 21–35.

Hempel, J., Kaiser, R., & Jörnvall, H. (1985) *Eur. J. Biochem. 153*, 13–28.

Hempel, J., Harper, K., & Lindahl, R. (1989) *Biochemistry 28*, 1160–1167.

Hempel, J., Nicholas, H., & Jörnvall, H. (1991) *Proteins: Struct., Funct., Genet.* (in press).

Holbrook, J., Liljas, A., Steindel, S. J., & Rossman, M. G. (1975) in *The Enzymes (3rd Edition)* (Boyer, P. D., Ed.) Vol. II, pp 191–268, Academic Press, Inc., New York.

Hsu, C. L., Tani, K., Fujiyoshi, T., Kurachi, K., & Yoshida, A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3771–3775.

Hsu, L. C., Bendel, R. E., & Yoshida, A. (1988) *Genomics 2*, 57–65.

Hsu, L. C., Chang, W.-C., & Yoshida, A. (1989) *Genomics 5*, 857–865.

Johansson, J., von Bahr-Lindström, H., Jeck, R., Woenckhaus, C., & Jörnvall, H. (1988) *Eur. J. Biochem. 172*, 528–533.

Jones, D. E., Jr., Brennan, M. D., Hempel, J., & Lindahl, R. (1988) *Proc. Natl. Acad. U.S.A. 85*, 1782–1786.

Kaiser, R., Holmquist, B., Hempel, J., Vallee, B. L., & Jörnvall, H. (1988) *Biochemistry 27*, 1132–1140.

Kok, M., Oldenhuis, R., van der Linden, M. P. G., Meulenberg, C. H. C., Kingma, J., & Witholt, B. (1989) *J. Biol. Chem. 264*, 5442–5451.

Lindahl, R. (1986) *Cancer Res. 46*, 2502–2506.

Loomes, K. M., Midwinter, G. G., Blackwell, L. F., & Buckley, P. D. (1990) *Biochemistry 29*, 2070–2075.

O'Connell, M., & Kelly, J. (1989) *Gene 84*, 173–180.

Pickett, M., Gwynne, D. I., Buxton, D. I., Elliot, R., Davies, R. W., Lockington, R. A., Scazzochio, C., & Sealy-Lewis, H. M. (1987) *Gene 51*, 217–226.

von Bahr-Lindström, H., Hempel, J., & Jörnvall, H. (1984) *Eur. J. Biochem. 141*, 37–42.

von Bahr-Lindström, H., Jeck, R., Woenckhaus, C., Sohn, S., Hempel, J., & Jörnvall, H. (1985) *Biochemistry 24*, 5847–5851.

Yin, S.-J., Vagelopoulos, N., Wang, S.-L., & Jörnvall, H. (1991) *FEBS Lett. 283*, 85–88.

Yoshida, A., Huang, I.-Y., & Ikawa, M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 258–261.