Characterization of the Coenzyme Binding Site of Liver Aldehyde Dehydrogenase: Differential Reactivity of Coenzyme Analogues†

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ABSTRACT: The mitochondrial isozyme of horse liver aldehyde dehydrogenase was labeled with brominated [5-(3-acetylpyridinio)pentyl]diphosphoadenosine. Specific labeling of a coenzyme binding region was proven by an enzymatic activity of the isozyme with the nonbrominated coenzyme derivative, optical properties of the complex, stoichiometry of incorporation, and protection against inactivation. A cysteine residue was selectively modified by the brominated coenzyme analogue and was identified in a 35-residue tryptic peptide. This cysteine residue corresponds to Cys-302 of the cytoplasmic isozyme and has earlier been implicated in disulfiram binding, confirming a position close to the active site. In contrast, the butyl homologue of the coenzyme analogue labels another residue of the mitochondrial isozyme. Thus, in the same isozyme, two residues are selectively reactive. They are concluded to be close together in the tertiary structure and to be close enough to the coenzyme binding site to be differentially labeled by coenzyme analogues differing only by a single methylene group.

Two main isozymes of mammalian liver aldehyde dehydrogenase (EC 1.2.1.3) have been purified, one cytoplasmic and one mitochondrial form [for review, see Weiner (1979)]. The isozymes have different affinity for both acetaldehyde and the coenzyme NAD (Eckfeldt et al., 1976), different sensitivity to inhibition by disulfiram (Eckfeldt et al., 1976), and clearly distinct but related structures (von Bahr-Lindström et al., 1982a; Hempel et al., 1983) that apparently do not cross-hybridize (Jörnvall, 1980). Alkylations done in the presence of disulfiram or directly with reactive coenzyme analogues have been utilized to map regions of functional importance in catalysis or coenzyme binding (von Bahr-Lindström et al., 1981; Hempel et al., 1982). In this way, inactivation of the protein with the NAD analogue [4-(3-acetylpyridinio)butyl]diphosphoadenosine has been shown (von Bahr-Lindström et al., 1981). A cysteine residue, presumably close to the catalytic site and associated with disulfiram binding, has also been identified (Hempel et al., 1982). The enzymatic mechanism of aldehyde dehydrogenase is proposed to involve a catalytic thiol(s) (Feldman & Weiner, 1972a; Buckley & Dunn, 1982), and the alkylations therefore support the general conclusions of functionally important cysteine residues.

The first complete primary structure of an aldehyde dehydrogenase, the human cytoplasmic isozyme (Hempel et al., 1984), recently became available. It allows comparisons with the horse enzyme (von Bahr-Lindström et al., 1984) and identifies the residue implicated in disulfiram binding as Cys-302. This residue is not identical with the one reactive with the butyl derivative of the coenzyme analogue (von Bahr-Lindström et al., 1981). However, these conclusions are based on labelings of different isozymes (mitochondrial for the coenzyme analogue and cytoplasmic for the disulfiram inhibition), and the isozymes are known to differ considerably (Hempel et al., 1983). Furthermore, only one of two alkylations with coenzyme analogues has been studied (von Bahr-Lindström et al., 1981), and the disulfiram effect is indirectly known [via carboxamidomethylation (Hempel et al., 1982)]. Therefore, is it essential to further determine the nature of the residues close to the active site.

In the present work, we identify in the horse mitochondrial isozyme the cysteine residue modified by a second coenzyme analogue. The coenzyme analogue now used is [5-[3-(bromoacetyl)pyridinio]pentyl]diphosphoadenosine, i.e., the pentyl derivative homologous to the butyl derivative previously used. The results complement those previously obtained with disulfiram and establish that Cys-302 is functionally important close to the active site. They also illustrate isozyme differences and show differential labeling of two residues by slightly different coenzyme analogues.

Materials and Methods

Enzymes and Coenzymes. Aldehyde dehydrogenase was prepared from horse liver (Eckfeldt et al., 1976; Feldman & Weiner, 1972b) with a DEAE-cellulose chromatography step to separate the two isozymes. After additional purification (Eckfeldt et al., 1976; Feldman & Weiner, 1972b; Greenfield & Pietruszko, 1977), the acidic [i.e., mitochondrial (Eckfeldt & Yonetai, 1976)] and basic [i.e., cytoplasmic (Eckfeldt & Yonetai, 1976)] isozymes were pure as judged by the presence of only one band on sodium dodecyl sulfate/polyacrylamide gel electrophoresis, by total compositions indistinguishable from those published (Eckfeldt et al., 1976), and by comparisons with known aldehyde dehydrogenase structures (Hempel et al., 1984; von Bahr-Lindström et al., 1984).

Enzymatic activity was determined at 25 °C in 0.12 M sodium pyrophosphate, pH 9, with 7 mM propanal and 0.06 mM NAD^+. NAD^+ and NADH were purchased from Boehringer (Mannheim, FRG) while [5-(3-acetylpyridinio)-pentyl]diphosphoadenosine, the corresponding reduced form (the 4,4-dihydropyridino derivative), the 3-(bromoacetyl) derivative, and the [^{14}C]carbonyl-labeled form (sp act. 0.16–1.26 Ci/mol) were prepared as described (Woenckhaus & Jeck, 1977).

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Coenzyme Labeling and Differential Carboxymethylation. The binding constant for [5-(3-acetylpyridinio)pentyl]diphosphoadenosine in the complex with the mitochondrial isozyme of aldehyde dehydrogenase was determined by equilibrium dialysis as described (Dietz et al., 1977; Kirschner et al., 1971). Inactivation of the enzyme with [5-(3-(bromoacetyl)pyridinio]pentyl]diphosphoadenosine was carried out in the same way as with the corresponding butyl derivative (von Bahr-Lindström et al., 1981; Woenckhaus et al., 1979). The inactivation was associated with incorporation of radioactivity when the ^{14}C-labeled coenzyme analogue was used (Woenckhaus & Jeck, 1977), and the protective effect by coenzyme and substrate during inactivation was measured as described (von Bahr-Lindström et al., 1981). Complete loss of enzymatic activity was obtained after incorporation of 0.5 mol of coenzyme/mol of enzyme subunit (von Bahr-Lindström et al., 1981). The inactivated enzyme was treated first with sodium borohydride to stabilize the coenzyme derivative bound to the protein (Jörnvall et al., 1975) and then with dithiothreitol and iodoacetate to yield complete carboxymethylation as described (von Bahr-Lindström et al., 1981). Esterase activity was followed by hydrolysis of *p*-nitrophenyl acetate (0.016 M) in 0.12 M barbital buffer, pH 8.0, containing *p*-nitrophenyl acetate. Fluoroescence measurements were made with a Perkin-Elmer fluorescence spectrophotometer, Model MPF 4. The effect of disulfiram (Antabuse, Dumex AS, Denmark) on the cytoplasmic isozyme was tested by differential carboxymethylation in the presence/absence of the drug (von Bahr-Lindström et al., 1981).

Peptide Analysis. The carboxymethylated protein was treated with CNBr, trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), or staphylococcal Glu-specific protease (EC 3.4.21.19) as described (von Bahr-Lindström et al., 1984), and the resulting fragments were separated on Sephadex G-50 fine in 30% acetic acid. Radioactive fractions were pooled and fractionated further by repeated reverse-phase high-performance liquid chromatography steps on μBondapak C_{18} (Waters) in 0.1% trifluoroacetic acid with an acetonitrile gradient (Hempel et al., 1984; von Bahr-Lindström et al., 1984).

Total compositions were determined with a Beckman 121M analyzer or with a Waters amino acid analysis system with precolumn derivatization of amino acids with orthophthalaldehyde. Manual sequence degradations were carried out with a DABITC[1] method (Chang et al., 1978) using byproducts to assist the identifications (von Bahr-Lindström et al., 1982b). Liquid-phase sequencer degradations were performed in a Beckman 890C instrument with a 0.1 M Quadrol peptide program and with glycine-precycled polybrene as carrier (Jörnvall & Philipson, 1980). Phenylthiohydantoin derivatives were identified by reverse-phase high-performance liquid chromatography (Zimmerman et al., 1977). Radioactive peptides were monitored by removal of aliquots (2% of the volume) of the extracts from the sequence degradations for measurements in a scintillation counter.

Results

Labeling of Horse Liver Mitochondrial Aldehyde Dehydrogenase with [5-[3-(Bromoacetyl)pyridinio]pentyl]diphosphoadenosine at a Specific Site. The specificity of labeling at the coenzyme binding site was judged by measurements of properties of the complex between the enzyme and the unbrominated or brominated coenzyme derivative. The

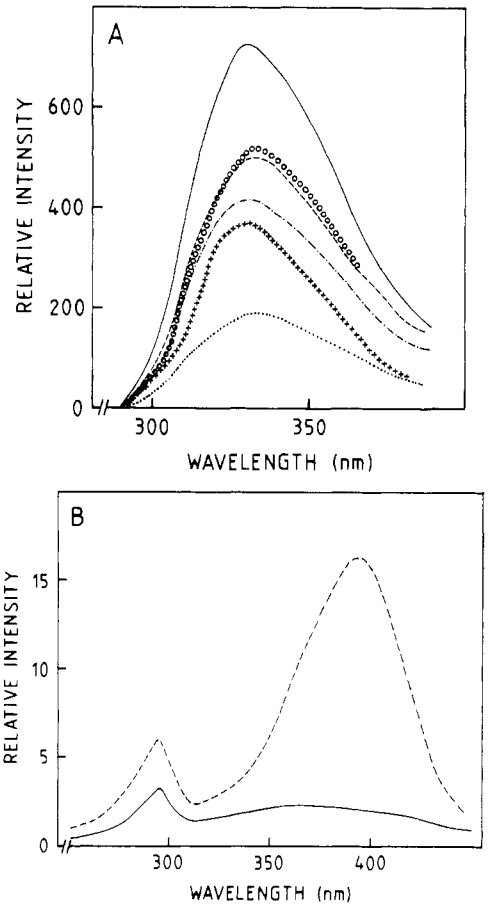

FIGURE 1: (A) Fluorescence emission spectrum after excitation at 290 nm of (—) aldehyde dehydrogenase, (-·-) aldehyde dehydrogenase–dihydronicotinamide adenine dinucleotide, (○) aldehyde dehydrogenase–[5-(3-acetylpyridinio)pentyl]diphosphoadenosine, (+) aldehyde dehydrogenase–[5-(3-acetyldihydropyridinio)pentyl]diphosphoadenosine, (--) aldehyde dehydrogenase inactivated with [5-[3-(bromoacetyl)pyridinio]pentyl]diphosphoadenosine, (···) aldehyde dehydrogenase inactivated with [5-[3-(bromoacetyl)pyridinio]-pentyl]diphosphoadenosine and reduced with dithionite. Protein and coenzyme concentration were 20 μM in 0.1 M phosphate buffer, pH 7, containing 0.1% mercaptoethanol. (B) Fluorescence excitation spectrum, emission wavelength 480 nm, of (—) aldehyde dehydrogenase inactivated with [5-[3-(bromoacetyl)pyridinio]pentyl]-diphosphoadenosine and (--) similarly inactivated and with dithionite-reduced enzyme. Concentrations were as in (A).

enzymatic activity of aldehyde dehydrogenase in the presence of the nonbrominated analogue has been reported (Jeck, 1977), and some enzymatic parameters are known (von Bahr-Lindström et al., 1981). The coenzyme-like binding is now further shown by fluorescence measurements. Thus, [5-(3-acetylpyridinio)pentyl]diphosphoadenosine is active as a coenzyme in the enzymatic reaction of horse liver mitochondrial aldehyde dehydroenase, and the dissociation constant for the binary complex between the enzyme and the analogue was determined to be 0.5 mM (at 25 °C) by equilibrium dialysis.

When the brominated analogue [5-[3-(bromoacetyl)-pyridinio]pentyl]diphosphoadenosine was used for labeling, the enzyme was inactivated. Loss of dehydrogenase activity paralleled the loss of esterase activity and was found to be pH dependent, indicating the participation of a group with a pK_a value of 8.25 (Sohn, 1978). The optical properties of the inactivated enzyme were now found to be similar to those of binary complexes with NAD. Thus, upon irradiation at 290 nm, the protein fluorescence at 340 nm decreased relative to that of the native enzyme (Figure 1A). Furthermore, treatment of the inactivated enzyme with sodium dithionite

[1] Abbreviations: DABITC, 4-(*N*,*N*-dimethylamino)azobenzene 4′-isothiocyanate; HPLC, high-performance liquid chromatography.

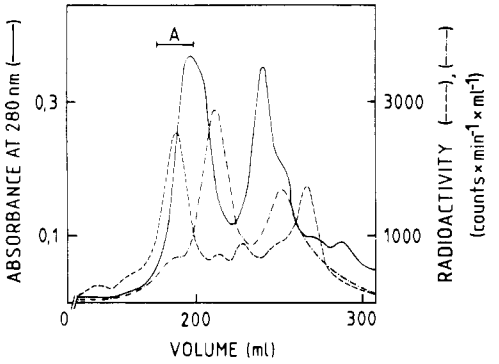

FIGURE 2: Sephadex G-50 chromatography of the tryptic peptides from aldehyde dehydrogenase modified by [^{14}C]carbonyl-labeled coenzyme analogues. (—) Absorbancy, (– –) radioactivity after modification with [5-[3-(bromoacetyl)pyridinio]pentyl]diphosphoadenosine (this work), and, for comparison (– · –), radioactivity after modification with the corresponding butyl analogue (von Bahr-Lindström et al., 1981). Column size was 1.5 × 200 cm; elution was with 30% acetic acid.

Table I: Total Compositions of the Labeled Peptide A and Its Subpeptides after Digestion of the Coenzyme-Modified Protein with Staphylococcal Glu-Specific Protease (Peptides E1 and E2) and Chymotrypsin (C1)[a]

	A	E1	E2	C1
Cys (CM)	1.1 (2)		1.6 (2)	1.5 (2)
Asx	5.3 (5)	3.7 (4)	1.4 (1)	1.4 (1)
Ser	2.8 (3)	1.6 (2)	1.3 (1)	1.2 (1)
Glx	4.4 (4)	1.3 (1)	3.0 (3)	2.0 (2)
Pro	1.2 (1)	nd (1)		
Gly	3.0 (3)		2.1 (3)	2.6 (3)
Ala	3.6 (5)	1.8 (2)	2.4 (3)	1.1 (1)
Val	2.0 (2)	1.7 (2)		
Met	0.6 (1)	0.6 (1)		
Ile	1.2 (2)	1.3 (2)		
Leu	1.4 (1)		1.2 (1)	
Phe	2.7 (3)		2.0 (3)	0.7 (1)
Trp	+ (1)	+ (1)		
His	0.4 (1)		0.7 (1)	
Arg	0.9 (1)		0.6 (1)	0.9 (1)
sum	35	16	19	12

[a] Values are molar ratios from acid hydrolysis, without corrections for destruction or slow release; Pro was not determined (nd), and Trp was estimated from peptide absorbance at 280 nm. Numbers within parentheses show the sums from the amino acid sequence.

yielded the reduced form (the 4,4-dihydropyridinio derivative) of the incorporated coenzyme analogue, as judged by the fluorescence excitation spectrum, which shows a transfer band at 290 nm (emission at 480 nm; Figure 1B). This was paralleled by similar changes for the complex of the native enzyme with NADH, where the emission maximum was increased and shifted to a shorter wavelength.

Purification of a Fragment Labeled by the Reactive Pentyl Coenzyme Analogue. The mitochondrial isozyme inactivated by ^{14}C-labeled [5-[3-(bromoacetyl)pyridinio]pentyl]diphosphoadenosine, reduced with sodium borohydride and carboxymethylated with nonradioactive iodoacetate, was digested with trypsin. The digest was fractionated by exclusion chromatography on Sephadex G-50 as shown in Figure 2. The results directly show that the labeled peptide is different from that previously modified by the corresponding butyl derivative (von Bahr-Lindström et al., 1981). For comparison, the elution pattern of radioactivity then obtained has also been transferred to Figure 2. The main radioactive peak now obtained, indicated by bar A in Figure 2, was further fractionated by consecutive steps of HPLC. Only one labeled peptide (A) was detected. Due to its size and relative insolubility, it was finally

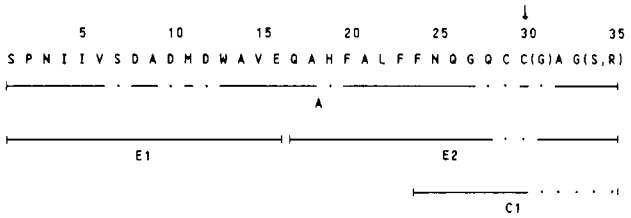

FIGURE 3: Amino acid sequence of the tryptic 35-residue peptide A labeled with the pentyl derivative of the coenzyme analogue. Positions of all peptides analyzed are shown, E1 and E2 corresponding to peptides obtained by digestion with the Glu-specific protease and C1 corresponding to a chymotryptic peptide. Solid lines indicate regions analyzed by sequencer degradations (cf. Table II) and/or manual DABITC degradations (7 positions in E1, 12 in E2, and 7 in C1), while dots indicate those parts of a peptide with not fully unambiguous residue identifications (low yield). Residues within parentheses are considered tentative because of limitations of material. Label was selectively recovered in the cysteine residue indicated by the arrow.

Table II: Results from Liquid-Phase Sequencer Degradations of Peptide A and Its Subpeptides after Digestion with Staphylococcal Glu-Specific Protease[a]

	A	E1	E2		A	E1	E2
S	+	+		F	0.8		2.6
P	0.5	1.3		A	0.7		2.0
N	1.1	1.5		L	0.4		1.5
I	1.3	2.8		F	0.4		2.2
I	1.4	3.0		F	0.5		2.2
V	1.5	2.0		N	<0.3		0.4
S		+		Q	<0.3		<0.3
D	<0.3	0.7		G	<0.3		0.5
A	1.1	1.4		Q			<0.3
D		0.7		C			
M	0.3	1.2		C	R		
D		0.6		G			0.4
W	0.6	0.9		A	<0.3		0.4
A	1.1	0.7		G	<0.3		<0.3
V	1.4	0.6		S	+		+
E	0.6	<0.3		R			
Q	<0.3		2.0	repetitive yield	97	88	96
A	0.7		1.9				
H			0.8				

[a] Values are nanomoles recovered. Identifications are by HPLC, complemented by radioactivity measurements, indicated by R when positive, for cysteine derivatives. Repetitive yields (%) are shown below each peptide.

recovered in low yield (approximately 10%).

Structure of the Fragment Labeled by the Reactive Pentyl Coenzyme Analogue. The amino acid composition of tryptic peptide A, radioactively labeled by the pentyl derivative of the coenzyme analogue, is shown in Table I, together with its fragments obtained by digestions of A with staphylococcal Glu-specific protease and chymotrypsin, respectively. The primary structure of the labeled tryptic peptide was determined by sequence analysis of all the fragments shown in Figure 3. The results from liquid-phase sequencer degradations are shown in Table II. The radioactivity from the labeled coenzyme analogue (50 cpm/nmol of peptide) was recovered in the residue corresponding to Cys-30 of the entire structure in Figure 3. No other radioactively labeled residue was recovered in tryptic peptide A or the other peptides indicated.

Structure of the Fragment Differentially Carboxymethylated in the Presence/Absence of Disulfiram. The effect of disulfiram on cysteine alkylation has earlier been examined (von Bahr-Lindström et al., 1981; Hempel et al., 1982). A large CNBr fragment of the cytoplasmic isozyme was found to be protected against labeling in the presence of disulfiram but was not identified (von Bahr-Lindström et al., 1981). These experiments were therefore now repeated, and a tryptic peptide, which is a constituent of the labeled CNBr fragment,

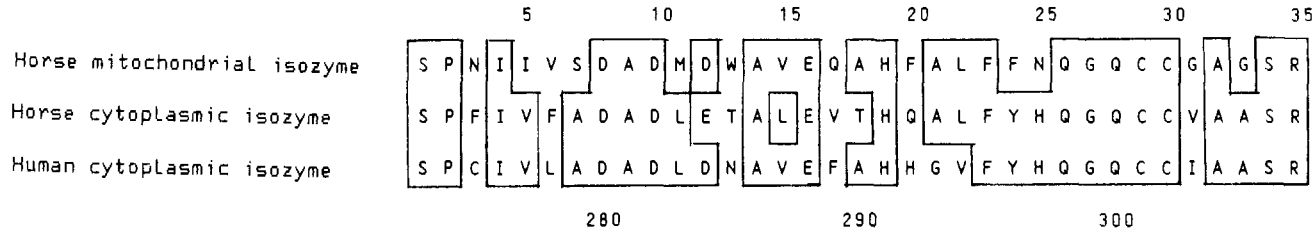

FIGURE 4: Structural comparisons of the homologous regions preferentially labeled in different isozymes of aldehyde dehydrogenase. The horse liver mitochondrial isozyme given in the top line was presently analyzed (and is shown disregarding the tentative nature of position 31), the cytoplasmic isozymes below are from von Bahr-Lindström et al. (1984) and Hempel et al. (1984), respectively. Top line numbers refer to positions in the peptide analyzed (cf. Figure 3); bottom line numbers refer to positions in the whole protein [cf. Hempel et al. (1984) and von Bahr-Lindström et al. (1984)]. The cysteine residue corresponding to position 30 in the segment shown is preferentially labeled with the pentyl derivative of the coenzyme analogue (present work) and with iodoacetamide (Hempel et al., 1982). Further, in both cases, this cysteine residue is protected against modification by the presence of coenzymes.

was purified. In the absence of disulfiram, the cysteine residues of this peptide (homologous to peptide A, Figure 3) had a 10-fold higher incorporation of radioactivity than the cysteine residues of other tryptic peptides (the radioactivity incorporated was 10^3 cpm/nmol vs. 10^2 cpm/nmol). Consequently, the most reactive cysteine residues in horse liver cytoplasmic aldehyde dehydrogenase appear to be in the tryptic peptide corresponding to the structure shown in Figure 3, supporting an accessible position of this segment in agreement with the labeling obtained with the coenzyme analogue. These results also prove that disulfiram protects identical positions in the horse and human enzymes (Hempel et al., 1982).

Discussion

Identification of the Cysteine Residue Labeled by the Pentyl Derivative of the Coenzyme Analogue. The labeling of the mitochondrial isozyme of horse liver aldehyde dehydrogenase is selective with the pentyl derivative (Figure 2), in the same way as previously shown for the corresponding butyl derivative (von Bahr-Lindström et al., 1981). The selective binding at the coenzyme binding site is shown by an enzymatic activity of the nonbrominated coenzyme derivative (Jeck, 1977), the optical properties of the complex (Figure 1), the stoichiometry of incorporation (Sohn, 1978), and the protection against inactivation (von Bahr-Lindström et al., 1981). The cysteine residue of the mitochondrial isozyme has now been identified (Figure 3) and corresponds to the disulfiram-sensitive cysteine residue of the cytoplasmic isozyme from human (Hempel et al., 1982) and horse liver (carboxymethylation above).

Differential Reactivity of Two Different Residues with Butyl and Pentyl Derivatives of Similar Coenzyme Analogues. Homologous coenzyme analogues, [4-(3-acetylpyridinio)butyl]diphosphoadenosine and [5-(3-acetylpyridinio)pentyl]diphosphoadenosine, label two different residues in the same isozyme. Modification with the butyl derivative has previously been reported (von Bahr-Lindström et al., 1981), and the pentyl derivative is presently shown (Figure 3) to label another residue (Figure 3). Both these modifications have now been studied in the same isozyme from one species, the mitochondrial isozyme from horse liver. Consequently, it may be concluded that the two residues labeled are likely to be close together in the tertiary structure, adjacent to the coenzyme binding site, and differentially accessible to the two coenzyme analogues, which deviate by only one methylene group.

The cysteine residue presently modified corresponds to Cys-302 (Hempel et al., 1984) and is identical with the cysteine residue implicated in the disulfiram reaction (Hempel et al., 1982, 1984). Consequently, we have now shown by direct evidence that Cys-302 is close to the coenzyme binding site, in agreement with the indirect evidence of an active site location from the disulfiram effect. A direct identification of the catalytic thiol still remains to be carried through, but the properties of Cys-302 make it a likely candidate. This residue appears to be close to the active site, important for catalytic function, labeled with reactive coenzyme analogues, and protected completely by NAD and partly by substrate, as evidenced from the present identification in relation to previous studies (Hempel & Pietruszko, 1981; Hempel et al., 1982, 1984).

Finally, it may be noticed that the differential reactivity of two residues with slightly different coenzyme analogues is not only found in aldehyde dehydrogenase but also in the enzyme of the preceding step of alcohol metabolism. Thus, alcohol dehydrogenase has two differentially reactive cysteine residues, far separated in primary structure but closely associated in the tertiary structure as deduced from differential labeling with coenzyme analogues (Jörnvall et al., 1975; Woenckhaus et al., 1979), which is in agreement with crystallographic data (Eklund et al., 1976). Although enzyme structures and mechanisms are completely different in alcohol dehydrogenases and aldehyde dehydrogenases, the parallel results illustrate the usefulness of slightly different analogues to map the active sites of enzymes.

Comparison of Isozyme Structures. The present identification of a reactive cysteine residue in mitochondrial horse liver aldehyde dehydrogenase (Figure 3) makes it possible to compare the same region in three different aldehyde dehydrogenases. Thus, this region has now been identified in the horse mitochondrial isozyme (this work) as well as in the cytoplasmic isozyme of both horse (von Bahr-Lindström et al., 1984) and man (Hempel et al., 1984). The corresponding structures are compared in Figure 4. The results show that the differences between the species are smaller than the differences between the mitochondrial and cytoplasmic isozymes in one species, in agreement with preliminary estimates from another region previously compared (Hempel et al., 1983).

Functionally, it is of interest that the whole area is well conserved in all structures, supporting the conclusion that Cys-302, corresponding to position 30 in Figure 4, is close to the active site and functionally important. It is also noticed (Figure 4) that a histidine residue, His-291, corresponding to position 19 in Figure 4, is strictly conserved. This may be of interest in relation to reports of the involvement of a histidine residue in the catalytic mechanism of aldehyde dehydrogenase (Takahashi et al., 1981). In any event, use of the reactive coenzyme analogues identifies two functionally important residues in aldehyde dehydrogenase, close together in space but nonadjacent in primary structure. The presence of two cysteine residues in the vicinity of the active site is compatible with the suggested mechanism of disulfiram inactivation (Vallari & Pietruszko, 1982; Kitson, 1982; Kitson, 1983), and arsenite inactivation (Deitrich, 1967). Thus, the structural

data help to characterize the coenzyme binding and correlate well with inhibitor studies.

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