Structural Relationships among Class I Isozymes of Human Liver Alcohol Dehydrogenase†

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ABSTRACT: The α subunit of human liver alcohol dehydrogenase has been submitted to structural analysis. Together with earlier work on the β and γ subunits, the results allow conclusions on the relationship of all known forms of the class I type of the enzyme. Two segments of the α subunit were determined; one was also reinvestigated in the β and γ subunits. The results establish 11 residue replacements among class I subunits in the segments analyzed and show that the α, β, and γ protein chains each are structurally distinct in the active site regions, where replacements affect positions influencing coenzyme binding (position 47; Gly in α, Arg in β and γ) and substrate specificity (position 48; Thr in α and β, Ser in γ). Residue 128, previously not detected in β and γ subunits, corresponds to a position of another isozyme difference (Arg in β and γ, Ser in α). The many amino acid replacements in alcohol dehydrogenases even at their active sites illustrate that in judgements of enzyme functions absolute importance of single residues should not be overemphasized. Available data suggest that α and γ are the more dissimilar forms within the family of the three class I subunits that have resulted from two gene duplications. The class distinction of alcohol dehydrogenases previously suggested from enzymatic, electrophoretic, and immunological properties therefore also holds true in relation to their structures.

Human liver alcohol dehydrogenase is a highly polymorphic enzyme. The isozymes are dimeric and have been subdivided into three classes on the basis of differences in sensitivity to inhibitors, as well as in substrate specificity, immunological properties, and electrophoretic migration (Strydom & Vallee, 1982).

Class I isozymes were those discovered first. Studies of their genetics suggested a scheme of three gene loci, coding for subunits α, β, and γ, which could combine dimerically to produce all of the then known isozymes (Smith et al., 1971). Many of these isozymes have since been purified and studied enzymatically (Bosron et al., 1983; Wagner et al., 1983). However, most studies concern the β and γ subunits, which are abundant in adult liver, whereas comparatively little is known about the α subunit, which is the main form expressed in fetal liver (Smith et al., 1971; Pikkarainen & Räihä, 1969; Murray & Motulsky, 1971). The introduction of an affinity medium for chromatographic purification (Lange & Vallee, 1976) greatly facilitated isolation of all isozymes and led to the recognition of three classes (Strydom & Vallee, 1982). Thus, besides class I isozymes, with α, β, and γ subunits that are basic in charge, class II isozymes with π subunits, which are less basic and less pyrazole sensitive (Li et al., 1977) were discovered, as well as class III isozymes with χ subunits, which are anodic forms with remarkably low ethanol dehydrogenase activity (Parés & Vallee, 1981).

The primary structures of the β and γ subunits (Hempel et al., 1984; Bühler et al., 1984a) and the structural differences between them were recently reported (Bühler et al., 1984b). Furthermore, the allelic variants constituting the "atypical" (von Wartburg et al., 1965) isozymes have been characterized (Jörnvall et al., 1984a; Bühler et al., 1984c). Thus, their β_2 subunit differs from β_1 by a single replacement (His instead of Arg at position 47, accounting for the altered enzymatic properties). Finally, structures from cloned cDNA for part (Duester et al., 1984) or all (Ikuta et al., 1985) of the β subunit have been reported.

In spite of this progress, no structural data are available for the third type, α, of the class I subunits. We report such data in the present study. We establish that, structurally, this fetal form is a typical member of class I, confirming the class distinction. The data obtained also separate the structure of α subunits from those of both β and γ, suggesting that the three subunits have evolved by two comparatively recent but different gene duplications. The replacements found at the active site of the α subunit are functionally important.

Materials and Methods

Protein. The homodimeric $\alpha\alpha$, $\beta\beta$, and $\gamma\gamma$ isozymes of human liver alcohol dehydrogenase were purified by affinity chromatography on CApGApp–Sepharose (Lange & Vallee, 1976). Protein purity was determined by gel electrophoresis in starch and in sodium dodecyl sulfate (SDS)–polyacrylamide. Sequence analysis showed that peptides from the $\gamma\gamma$ preparations sometimes were contaminated with β peptides, demonstrating incomplete separation of isozymes in the starting material. Probable contaminants are $\beta\gamma$ in the $\gamma\gamma$ preparations. It should be emphasized, however, that this contamination did not cause problems in interpretation when, as is the

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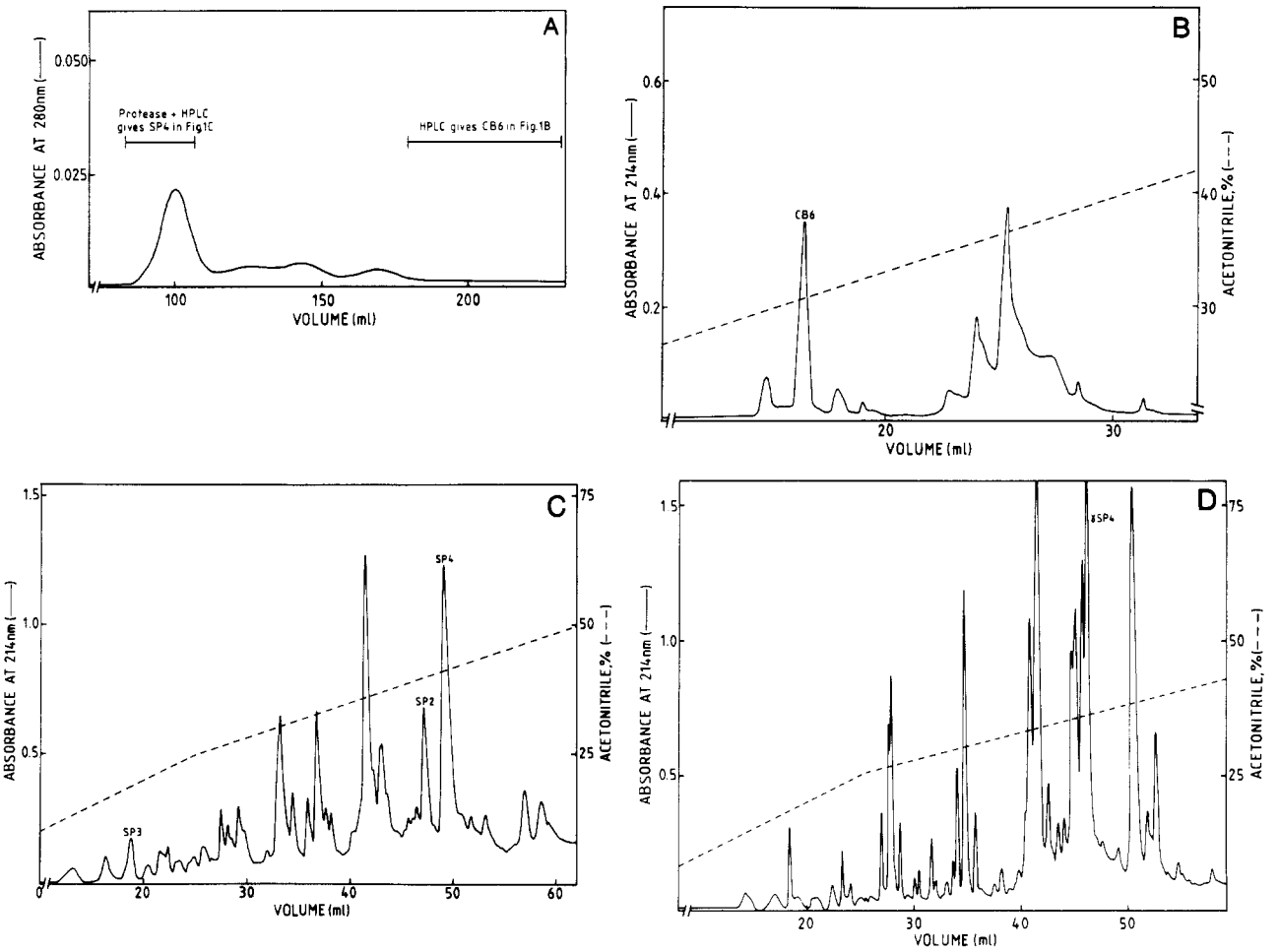


FIGURE 1: Purification of the α peptides studied. (A) Fractionation of the CNBr fragments on Sephadex G-50 (1.4 × 180 cm) in 30% acetic acid. (B) Purification of the CNBr fragment (CB6) corresponding to positions 41–57, containing an active site segment. Reverse-phase high-performance liquid chromatography on μBondapak C_{18} in 0.1% trifluoroacetic acid with a gradient of acetonitrile as indicated. The material applied corresponds to the fraction of small peptides from (A). (C) Separation of the peptides obtained after digestion of the largest CNBr fragment with Glu-specific staphylococcal protease. Prefractionation of CNBr fragments by Sephadex G-50 chromatography in (A) and final purification of enzymatic peptides by reverse-phase high-performance liquid chromatography as in (B). Peptides analyzed were selected from known strutures of the β and γ subunits and are indicated by SP and a number to reflect the order in Table IB. (D) For comparison, the same separation as in (C) but with the $\gamma\gamma$ isozyme instead of the $\alpha\alpha$ isozyme.

case here, the peptides that are analyzed are sufficiently long also to contain positions with exchanges to prove the origins from different isozymes. All isozymes were reduced with dithiothreitol in 6 M guanidine hydrochloride and ^{14}C-carboxymethylated with labeled iodoacetate (Jörnvall et al., 1984a).

Structural Analysis. The carboxymethylated proteins were cleaved with CNBr, and the resultant fragments were fractionated by Sephadex G-50 chromatography in 30% acetic acid (Jeffery et al., 1984). For subcleavages of large fragments, fractions were digested with Glu-specific staphylococcal protease in 0.1 M ammonium bicarbonate (approximately 50 nmol of fragment per 25 μg of enzyme in 200 μL of buffer). For final analysis, all peptides were purified by reverse-phase high-performance liquid chromatography on μBondapak C_{18}, utilizing linear gradients of acetonitrile in 0.1% trifluoroacetic acid (Hempel et al., 1984).

Amino acid compositions were determined with a Beckman 121M analyzer after acid hydrolysis (24 h, 110 °C, 6 M HCl/0.5% phenol). Sequential degradations were carried out with the (dimethylamino)azobenzene isothiocyanate method or in a Beckman 890D liquid-phase sequencer. Phenylthiohydantoins were identified by high-performance liquid chromatography (Jeffery et al., 1984).

RESULTS

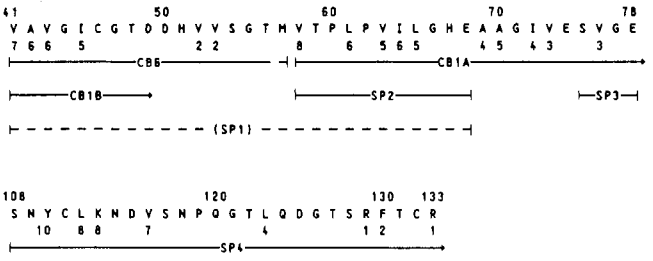
Analysis of the α Subunit. The homodimeric $\alpha\alpha$ isozyme of human liver alcohol dehydrogenase was carboxymethylated and cleaved with CNBr. Separation of the fragments by Sephadex G-50 chromatography in 30% acetic acid gave a pattern of fractions (Figure 1A) that generally resembled that from similarly treated $\beta\beta$ and $\gamma\gamma$ isozymes (Hempel et al., 1984; Bühler et al., 1984a), suggesting overall similarities.

Degradation by liquid-phase sequencer revealed that the material in the first chromatographic peak (Figure 1A) contained the largest CNBr fragment CB1A, corresponding to positions 58–209 in the homologous isozymes; there was also a minor amount of this fragment extended at the N-terminal end, CB1B, derived from nonquantitative cleavage at the preceding Met-57. These peptides contain Cys-46 and His-67, two of the active site Zn ligands. Degradation for 20 steps gave a tentative structure for the major component CB1A starting at position 58 (Table IB) and the minor component CB1B starting at position 41. These results suggest that in the α subunit the residue at position 57 is methionine. Therefore, cleavage with CNBr should also give rise to a 17-residue fragment containing residues 41–57. This fragment was purified by reverse-phase high-performance liquid chromatography (Figure 1B) from the last pool of the Sephadex

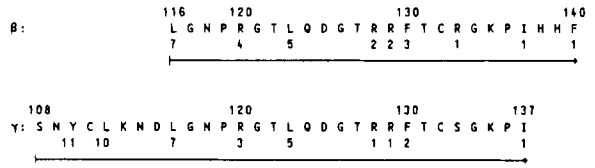
Table I: Results of Structural Analysis of Peptides Covering the Two Regions Analyzed

peptide	CB6	SP2	CB1A	SP3	SP4
	(A) Total Compositions of α Peptides[a]				
Cys	0.8 (1)		6.4 (8)		1.5 (2)
Asx	1.9 (2)		12.9 (11)		5.6 (6)
Thr	1.8 (2)	1.0 (1)	10.4 (11)		4.6 (5)
Ser	0.9 (1)		10.7 (11)	0.9 (1)	4.7 (5)
Glx		1.0 (1)	13.2 (10)	1.0 (1)	4.3 (4)
Pro		1.8 (2)	10.6 (10)		2.2 (2)
Gly	2.9 (3)	1.2 (1)	15.4 (18)	1.1 (1)	3.3 (3)
Ala	1.0 (1)		11.4 (11)		
Val	3.6 (4)	1.6 (2)	14.7 (18)	1.0 (1)	2.7 (3)
Hse	0.3 (1)		0.4 (1)		
Ile	1.0 (1)	0.9 (1)	8.2 (9)		2.0 (2)
Leu		1.7 (2)	10.3 (10)		3.2 (3)
Tyr			2.7 (3)		1.9 (2)
Phe			5.3 (5)		2.6 (3)
Lys			8.5 (9)		2.2 (2)
His	0.9 (1)	0.8 (1)	3.3 (3)		1.9 (2)
Arg			4.2 (4)		2.9 (3)
sum	17	11	152	4	47

(B) Amino Acid Sequences of Two α Segments[b]



(C) Sequencer Degradations of the Second Segment in β and γ Subunits[c]



[a] Analytical values after acid hydrolysis are given in molar ratios without corrections for destruction, slow release, or impurities, with values from sequence analysis within parentheses. [b] The two α segments are the one around the active site zinc liganding Cys-46 and His-67 (top) and the one corresponding to parts of a missing structure in distantly related alcohol dehydrogenases (bottom). Numbers above the residues show positions in the β subunit of human and horse liver alcohol dehydrogenase; numbers below the residues show recoveries in nanomoles of relevant phenylthiohydantoin derivatives upon sequencer degradation of 10 (α and β) to 15 (γ) nmol of peptide. Peptides were purified as shown in Figure 1. The top sequence is based on degradations of two major CNBr fragments, CB6 and CB1A. The overlap is established, apart from homology with the other isozymes (Figure 2), by recovery of two fragments in minor yield, CB1B and SP1. CB1B is an N-terminally extended form of CB1A; it was monitored and detected by sequence analysis of the first pool from Sephadex prefractionation of CB1A + CB1B (Figure 1A). SP1 is derived from that pool by digestion with Glu-specific staphylococcal protease (dashed line indicates low recovery). The bottom sequence was determined by sequencer degradation of the major large peptide (SP4 in Figure 1C) from the protease digestion of CB1A. [c] The β subunit is on the top, and the γ subunit is on the bottom. The peptides were obtained as shown in Figure 1D, i.e., in the same way as for SP4 from the α subunit, by subdigestion of the largest CNBr fragment (cf. Figure 1A) from isozymes $\beta\beta$ and $\gamma\gamma$ with Glu-specific staphylococcal protease. In the case of the β subunit, the peptide was obtained in a shortened form, because of an extra cleavage at Asp-115, in agreement with the tendency to cleavage at some Asp residues by the protease. The degradations prove the presence of Arg at position 128 in both β and γ. Total compositions have been analyzed before (Hempel et al., 1984; Bühler et al., 1984a) and support the Arg assignment. Similarly, the residue differences at position 133 prove the β and γ assignments of the peptides.

fractionation (Figure 1A), giving peptide CB6 (Table IB). Structural analysis of this fragment, together with the initial sequencer degradation, yielded the active site region shown in Table IB, positions 41–78, containing the two zinc ligands Cys-46 and His-67, as well as residues 48 and 51 that participate in a hydrogen-bonding system to the nicotinamide ribose of the coenzyme (Eklund et al., 1982).

Redigestion of the large CNBr fragment, CB1A, in the first pool (Figure 1A) should yield peptides corresponding to the longest deleted region in yeast alcohol dehydrogenase (or inserted region in the mammalian enzyme) (Jörnvall et al., 1978). CB1A was therefore digested with Glu-specific staphylococcal protease, and the mixture was resolved by reverse-phase high-performance liquid chromatography, as shown in Figure 1C. Structural analysis of the largest fragment obtained (SP4, Table I) conclusively establishes that a serine residue is present in the α subunit at the position corresponding to residue 128 in the β subunit.

Analysis of the β and γ Subunits. The detection of a residue corresponding to position 128 in the α subunit prompted reinvestigation of this position in the β and γ subunits, where no such residue had been found (Bühler et al., 1984a,b; Hempel et al., 1984). Hence, the $\beta\beta$ and $\gamma\gamma$ isozymes were treated in the same manner as the $\alpha\alpha$ isozyme to produce the largest CNBr fragment, corresponding to positions 41–209. This was then redigested with the Glu-specific staphylococcal protease, and the peptide corresponding to positions 108–154 was purified by reverse-phase high-performance liquid chromatography (Figure 1D, γSP4). Sequencer analyses (Table IC) reveal that both β and γ subunits contain an arginine residue at position 128.

Discussion

Comparisons between Class I Isozyme Structures. The results allow the first structural comparisons of all three class I subunits. One segment analyzed contains Cys-46 and His-67, two of the ligands to the catalytic zinc atom, residues 48 and 51, which participate in a hydrogen-bonding system with the coenzyme (Eklund et al., 1982), and residue 47, which interacts with the pyrophosphate group of the coenzyme (Lange et al., 1975). At position 47, a His → Arg substitution in mutants of the yeast enzyme results in altered enzymatic activity (Wills & Jörnvall, 1979). Similarly, in the "atypical" enzyme from human liver an Arg → His substitution occurs at the same position (Jörnvall et al., 1984a; Bühler et al., 1984c). However, this whole region represents the most conserved segment in the distantly related yeast alcohol dehydrogenase (Jörnvall et al., 1978) and liver sorbitol dehydrogenase (Jörnvall et al., 1984b).

The other region analyzed corresponds to a large part of the segment previously identified as missing (positions 119–139) in the yeast enzyme (Jörnvall et al., 1978) and as missing or greatly different in sorbitol dehydrogenase (Jörnvall et al., 1984b; Eklund et al., 1985). This region contains position 128 not previously detected in the human β and γ alcohol dehydrogenase subunits (Hempel et al., 1984; Bühler et al., 1984a). It is now established to be Arg in β, in agreement with recent data for the β-cDNA (Ikuta et al., 1985), Arg in γ, and Ser in α. This position therefore corresponds to another isozyme difference (Figure 2), and the results show that—relative to the horse liver enzyme—there is no deletion or insertion in the three class I human subunits.

Residue differences are not the same in α and γ; α appears to have the most and β the least number of the unique residues (Figure 2). However, by any pairwise comparison the three

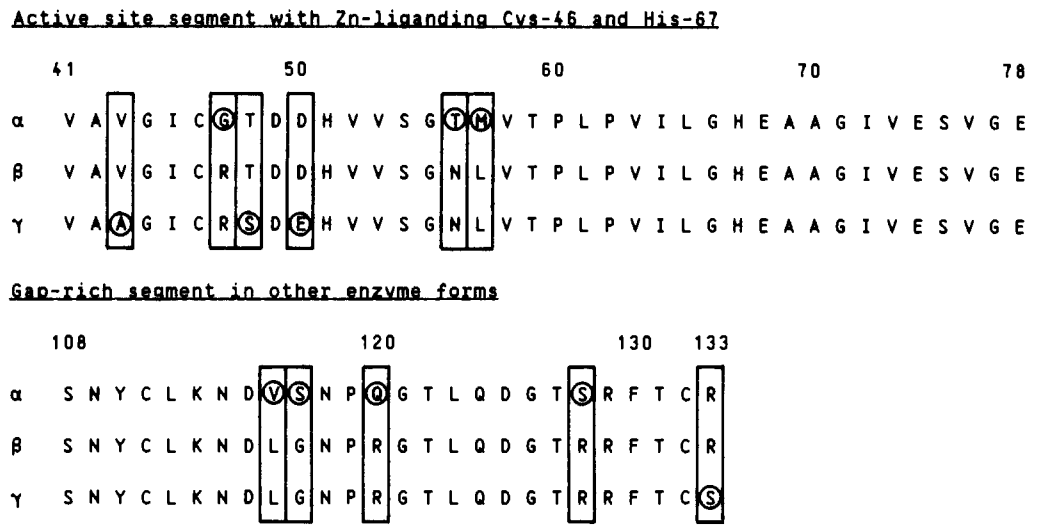


FIGURE 2: Comparisons of class I isozyme structures. Structures for all three subunits (α, β, γ) of class I isozymes around the conserved active site zinc liganding Cys-46 and His-67 (top) and in a segment (bottom) corresponding to one with long deletions/insertions in distantly related alcohol dehydrogenases. Data for the structures from Table I, Hempel et al. (1984), and Bühler et al. (1984a). Data for correlation with the distantly related enzymes from Jörnvall et al. (1978, 1984b) and Eklund et al. (1985). Numbers refer to residue positions. Positions showing isozyme differences are boxed; isozyme-unique residues are circled. In the two segments compared, the β alternative is either that of α or γ.

subunits differ considerably. Two separate gene duplications can account for these characteristics, explaining the three subunits of class I.

The finding that the two segments compared for all three subunits contain a nearly equal number of substitutions (Figure 2) was not expected; one segment is the active site region, which is usually the one that is most conserved when related forms of an enzyme are compared. The other segment corresponds to a region in which deletions/insertions have been encountered (Jörnvall et al., 1978, 1984b). The analysis of the α subunit adds importantly to the resolution of the relationships (Figure 2).

Functionally, the many substitutions in the active site segment of alcohol dehydrogenases illustrate that the absolute role of single residues in enzymes should not be overemphasized. Cys-46, Arg-47, Ser-48, and His-51 in the horse liver alcohol dehydrogenase have been assigned functional roles (Eklund et al., 1976, 1982). They are either exchanged in the α subunit of the human enzyme or are in a variable region (Figure 2). The α subunit does not have a basic residue at position 47 for direct interaction with the coenzyme pyrophosphate (Lange et al., 1975), but instead there is a glycine residue (Figure 2). However, this replacement is identical with one in the distantly related sorbitol dehydrogenase (Jörnvall et al., 1984b) and is one that also fits the model for coenzyme binding (Eklund et al., 1985). The frequency of exchanges in the active site structures also suggests enzymatic differences between the isozymes, in agreement with direct observations (Smith et al., 1971; Bosron et al., 1983; Wagner et al., 1983).

Registry No. Alcohol dehydrogenase, 9031-72-5.

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Articles

3-(Bromoacetyl)chloramphenicol, an Active Site Directed Inhibitor for Chloramphenicol Acetyltransferase†

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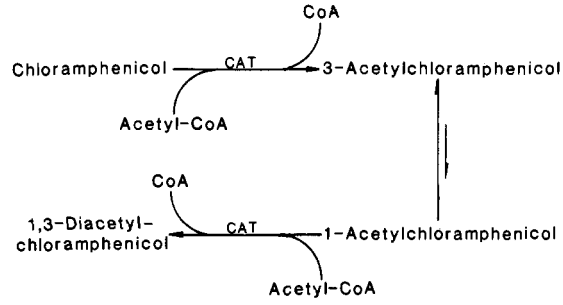
ABSTRACT: Bacterial resistance to the antibiotic chloramphenicol is normally mediated by chloramphenicol acetyltransferase (CAT), which utilizes acetyl coenzyme A as the acyl donor in the inactivation reaction. 3-(Bromoacetyl)chloramphenicol, an analogue of the acetylated product of the forward reaction catalyzed by CAT, was synthesized as a probe for accessible and reactive nucleophilic groups within the active site. Extremely potent covalent inhibition was observed. Affinity labeling was demonstrated by the protection afforded by chloramphenicol at concentrations approaching K_m for the substrate. Inactivation was stoichiometric, 1 mol of the inhibitor covalently bound per mole of enzyme monomer, with complete loss of both the acetylation and hydrolytic activities associated with CAT. N^3-(Carboxymethyl)histidine was identified as the only alkylated amino acid, implicating the presence of a unique tautomeric form of a reactive imidazole group at the catalytic center. The proteolytic digestion of CAT modified with 3-(bromo[^{14}C]-acetyl)chloramphenicol yielded three labeled peptide fractions separable by reverse-phase high-pressure liquid chromatography. Each peptide fraction was sequenced by fast atom bombardment mass spectrometry; the labeled peptide in each case was found to span the highly conserved region in the primary structure of CAT, which had been tentatively assigned as the active site. The rapid, stoichiometric, and specific alkylation of His-189, taken together with the high degree of conservation of the adjacent amino acid residues, strongly suggests a central role for His-189 in the catalytic mechanism of CAT.

Chloramphenicol acetyltransferase (CAT)[1] (EC 2.3.1.28) catalyzes the *O*-acetylation of the antibiotic chloramphenicol in both Gram-positive and Gram-negative organisms [reviewed by Shaw (1983)]. Whereas chloramphenicol binds to the 50S subunit of bacterial ribosomes and inhibits the peptidyl transferase reactions (Traut & Monro, 1964), acetylation of the antibiotic prevents ribosome binding (Shaw & Unowsky, 1968), thus confering the phenotype of chloramphenicol resistance. Chloramphenicol possesses two hydroxyl groups (see Figure 1), one or both of which may be acetylated in a reaction that is dependent on acetyl coenzyme A (acetyl-CoA) as the acyl donor (see Scheme I). Following the initial acetylation of the primary hydroxyl group (C_3 of the propanediol side chain, Figure 1), a nonenzymic and pH-dependent isomerization occurs (Nakagawa et al, 1979), exposing the same hydroxyl to reacetylation and formation of the 1,3-diacetyl derivative. The mono- and diacetylated products are devoid of antibiotic activity.

Three classes of chloramphenicol acetyltransferase have been detected in Gram-negative bacteria and have been classified as types I, II, and III (Foster & Shaw, 1973; Gaffney et al., 1978). The type III variant has been the focus of mechanistic

Scheme I



investigations (Kleanthous & Shaw, 1984) and has yielded crystals that are suitiable for the determination of a high-resolution structure by X-ray diffraction methods (A. Leslie and D. Blow, personal communication). The complete amino acid and DNA sequences have been determined for the type I variant of CAT (Shaw et al., 1979; Alton & Vapnek, 1979; Marcoli et al., 1980), and the gene for the type III enzyme

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[1] Abbreviations: CAT, chloramphenicol acetyltransferase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; TSE buffer, 50 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl and 0.1 mM EDTA; 3-BrCH_2COCm, 3-(bromoacetyl)chloramphenicol; 3-BrCH_2CO*Cm, 3-(bromoacetyl)[^{14}C]chloramphenicol; 3-BrCH_2*COCm, 3-(bromo[^{14}C]acetyl)-chloramphenicol.

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