of a Cl^--translocating ATPase.

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Evidence for Reactivity of Serine-74 with *trans*-4-(*N*,*N*-Dimethylamino)cinnamaldehyde during Oxidation by the Cytoplasmic Aldehyde Dehydrogenase from Sheep Liver

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ABSTRACT: A nucleophilic group in the active site of aldehyde dehydrogenase, which covalently binds the aldehyde moiety during the enzyme-catalyzed oxidation of aldehydes to acids, was acylated with the chromophoric aldehyde *trans*-4-(*N*,*N*-dimethylamino)cinnamaldehyde (DACA). Acyl-enzyme trapped by precipitation with perchloric acid was digested with trypsin, and the peptide associated with the chromophoric group was isolated and shown to be Gln-Ala-Phe-Gln-Ile-Gly-Ser-Pro-Trp-Arg. After redigestion with thermolysin, the chromophore was associated with the C-terminal hexaresidue part. If the chromophore is attached to this peptide, serine would be expected to bind the aldehyde and lead to the required acylated derivative. Differential labeling experiments were performed in which all free thiol groups on the acylated enzyme were blocked by carboxymethylation. The acyl chromophore was then removed by controlled hydrolysis and the protein reacted with [^{14}C]iodoacetamide. No ^{14}C-labeled tryptic peptides were isolated, suggesting that the sulfur of a cysteine cannot be the acylated residue in the precipitated acyl-enzyme.

Aldehyde dehydrogenase catalyzes the irreversible oxidation of a wide variety of aldehyde substrates (MacGibbon et al., 1977a,b) including the chromophoric aldehyde *trans*-4-(*N*,*N*-dimethylamino)cinnamaldehyde (DACA)[1] (Buckley & Dunn, 1982) by the ordered pathway shown in Scheme I. For DACA at pH 6, since the rate of hydrolysis of the acyl-enzyme

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[1] Abbreviation: DACA, *trans*-4-(*N*,*N*-dimethylamino)cinnamaldehyde.

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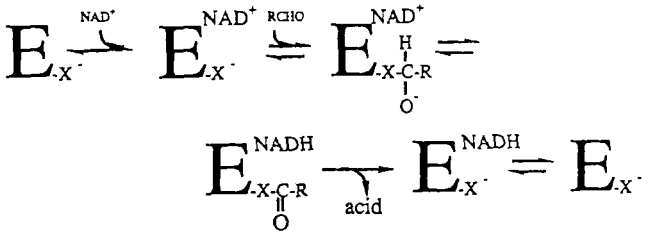
Scheme I



is slow, the concentration of the acyl-enzyme intermediate builds up and can be directly observed. This acyl-enzyme intermediate has a λ_{max} at 463 nm, a significantly longer wavelength than either reactants (NAD^+ λ_{max} = 260 nm; DACA λ_{max} = 398 nm) or products [NADH λ_{max} = 340 nm; *trans*-4-(*N*,*N*-dimethylamino)cinnamate ion λ_{max} = 323 nm] (Buckley & Dunn, 1982).

There is general agreement that the mechanism for the enzyme-catalyzed reaction is as shown in Scheme I (Dickinson, 1986; Blackwell et al., 1987, 1988); however, direct experimental evidence of the identity of the nucleophilic group on the enzyme (shown as $-X^-$) which covalently binds the aldehyde is lacking. It has often been assumed, by analogy with glyceraldehyde-3-phosphate dehydrogenase (Harris & Waters, 1976), that X is sulfur and that the aldehyde binds to a cysteine on the enzyme. Much attention has therefore been directed toward the effect of sulfhydryl reagents on the activity of the enzyme. Certainly when cytoplasmic aldehyde dehydrogenase is modified by disulfiram, there is a dramatic decrease in the enzyme activity (Kitson, 1978, 1982, 1983). However, the activity is not reduced to zero even in the presence of excess reagent, and mitochondrial aldehyde dehydrogenases are relatively unaffected by modification with disulfiram. Other sulfhydryl modifying reagents such as *p*-(chloromercuri)benzoate (Motion et al., 1984) and 2,2-dithiodipyridine (Kitson, 1982, 1986) can cause activation of cytoplasmic aldehyde dehydrogenase.

Conclusions based on the effects of sulfhydryl reagents rely on the specificity of the reagent and specific group protection by substrates, inhibitors, or substrate analogues. For an enzyme like aldehyde dehydrogenase with 36 SH groups per tetramer it is difficult to ensure that observed effects are due to reaction with an active site group. On the other hand with DACA as a substrate, the long-lived acyl-enzyme intermediate that is formed on the reaction pathway must have the chromophoric group attached to a nucleophilic group in the active site (Buckley & Dunn, 1982). We report experiments in which the DACA-labeled acyl-enzyme intermediate is trapped by rapid precipitation of the enzyme. After tryptic digestion, a single labeled peptide is located and sequenced which contains a serine residue. Carboxymethylation of the denatured acyl-enzyme intermediate, followed by hydrolysis of the acyl group and reaction with [^{14}C]iodoacetamide, gave no evidence for a cysteine as the active site nucleophile.

Experimental Procedures

Materials. Aldehyde dehydrogenase was purified according to Dickinson et al. (1981) with the inclusion of a pH gradient step to remove any mitochondrial contamination. Protein concentration was calculated by assuming that $^{1\%}A_{1cm}$ = 11.3 at 280 nm (Dickinson et al., 1981). Before use the enzyme was thoroughly dialyzed against several changes of degassed 50 mM phosphate buffer, pH 6.0, containing 0.3 mM EDTA, to remove dithiothreitol. (Dithiothreitol catalyzed the removal of the chromophore.) [^{14}C]Iodoacetamide was purchased from Aldrich Chemical Co. and diluted to a specific activity of 1.96 $\times$ 10^{12} counts min^{-1} mol^{-1} in the counting system used. DACA [*trans*-4-(*N*,*N*-dimethylamino)cinnamaldehyde] was obtained from Sigma and resublimed under reduced pressure before use. All other reagents and chemicals were of the highest purity available.

Acyl-Enzyme Formation, Isolation, and Digestion. An assay mixture (3 cm^3) containing 20 μM aldehyde dehydrogenase, 0.3 mM EDTA, and 1 mM NAD^+ in 50 mM phosphate buffer, pH 6.0, was incubated at 25 °C. Then, 25 μL of a stock solution of DACA in acetonitrile was added to give a DACA concentration in the assay of 30 μM. After approximately 30 s 70% perchloric acid (sufficient to give 3% by volume) was added to precipitate the enzyme, and the denatured protein was washed at least four times with water and redissolved in 8 M urea to give a protein concentration of 10 mg/mL (47 μM tetramer concentration, assuming a molecular weight of 212000). The mixture was then carboxymethylated with iodoacetate at 37 °C for 2 h in the dark, with 20% excess over enzymic thiols. Reagents were dialyzed out against several changes of 0.1 M ammonium bicarbonate at 4 °C. A control was also included in tandem following exactly the above procedure but with the omission of NAD^+. A tryptic digest was prepared by incubation with TPCK-trypsin (50:1 w/w) for 4 h in 0.1 M ammonium bicarbonate at 37 °C.

The acyl-enzyme was also trapped by precipitation with ethanol or by denaturation in saturated urea. In the latter case the reaction mixture was passed down a Bio-Gel P-6 column (0.7 cm $\times$ 12 cm) equilibrated in 8 M urea. Regardless of the method of trapping, the λ_{max} of the chromophore was 412 nm (in 8 M urea).

Peptide Purification. After tryptic digestion the material was freeze-dried and redissolved in 0.1 M ammonium bicarbonate. The peptides (approximately 20 mg/mL) were then loaded on a Superose 12 gel filtration column (10 mm $\times$ 30 mm) and eluted with 0.1 M ammonium bicarbonate with a flow rate of 0.40 mL/min. Fractions were monitored at 280 nm and manually collected in approximately 1-mL aliquots; the absorption spectrum of each was measured on a HP8452 diode array spectrophotometer. For reverse-phase chromatography fractions were loaded directly onto a Waters Associates Radial-Pak 8MBC1810μ cartridge preequilibrated in 0.1 M ammonium bicarbonate. A gradient was run to a mixture of acetonitrile, 2-propanol, and 0.1 M ammonium bicarbonate (1:1:1 v/v) over 1 h, and the eluate was monitored at 220 nm. Fractions were again collected manually ($\sim$1 mL), and the absorption spectrum of each was determined.

Redigestion with Thermolysin. In one experiment thermolysin ($\sim$18% by weight) was added to the peak fraction from the gel column which showed the 408-nm colored label (in 0.1 M ammonium bicarbonate). The sample was left for 10 min at room temperature and then loaded onto the HPLC column. The total amount of material loaded was approximately 8 mg. The same gradient system was used on the HPLC column, and the fractions were collected and measured as before.

Differential Labeling with [^{14}C]Iodoacetamide. The acyl intermediate was isolated as described above, but after carboxymethylation the acyl-enzyme (10 mg) was passed down a Bio-Gel P-6 column, which had been equilibrated with 8 M urea, in order to remove unreacted iodoacetate. The acyl-enzyme (10 mg mL^{-1}) was then incubated at 37 °C in the presence of excess [^{14}C]iodoacetamide (130 μM), and the spontaneous disappearance of the colored label was monitored. After 72 h the 412-nm peak (in urea) had dropped to 57% of its initial value. All reagents were then removed by dialysis, the material was digested with trypsin, and the peptides were

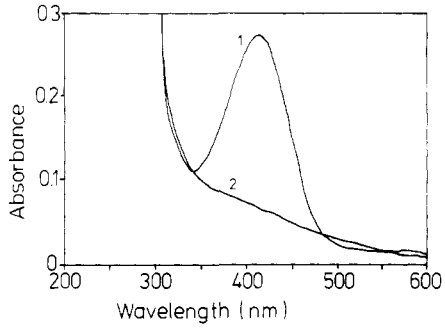


FIGURE 1: Spectrum 1 is the spectrum in 8 M urea of the denatured acyl-enzyme intermediate, prepared by perchloric acid precipitation of the enzyme in an assay mixture containing DACA, NAD^+, and aldehyde dehydrogenase at pH 6.1 as described under Experimental Procedures. Spectrum 2 is the spectrum in 8 M urea of denatured enzyme from a control experiment in which the conditions were identical with those in spectrum 1 except that NAD^+ was omitted from the assay mixture.

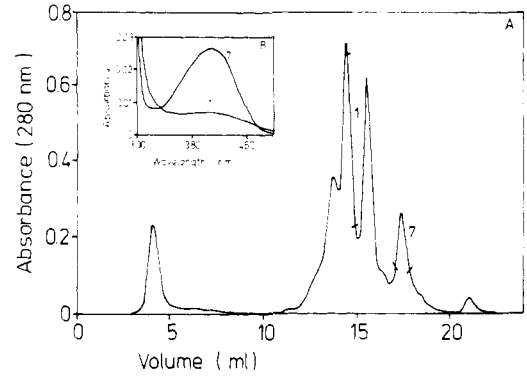


FIGURE 2: (A) Gel filtration profile of the tryptic peptides, from acyl-enzyme trapped on the dehydrogenase pathway with DACA as substrate, when eluted from a Superose 12 column with 0.1 M ammonium bicarbonate, as described under Experimental Procedures. (B) Absorbance spectra of fractions 1 and 7 from this column.

subjected to gel filtration as before and assayed for radioactivity. A similar experiment was carried out where the label was partially removed with ammonia before addition of excess [^{14}C]iodoacetamide. In each case a control experiment was carried out but with DACA omitted at the beginning.

Sequencing. All peptides were sequenced on the Applied Biosystems Model 470A protein sequencer with an associated Applied Biosystems reversed-phase HPLC 120A.

Results and Discussion

Trapping of the DACA-Labeled Acyl-Enzyme Intermediate. After mixing NAD^+, DACA, and ALDH at pH 6.1 and waiting 30 s for significant amounts of the acyl-enzyme intermediate to build up (Dunn & Buckley, 1985), the enzyme was rapidly precipitated with perchloric acid. Under these conditions the rate of hydrolysis of the *trans*-4-(*N*,*N*-dimethylamino)cinnamoyl-enzyme intermediate is slow, and the acyl group was effectively trapped on the protein surface. The precipitated protein, on rinsing with distilled water, gave a distinct yellow precipitate, due to the *trans*-4-(*N*,*N*-dimethylamino)cinnamoyl chromophore, which on being dissolved in 8 M urea showed an absorption peak with a λ_{max} at 412 nm (Figure 1). If it is assumed that the chromophore has an extinction coefficient similar to that of DACA and similar model compounds (i.e., around 30000 L mol^{-1} cm^{-1}), the percentage yield of labeled material is approximately equal to the total tetramer concentration. Thus one active site per tetramer has been acylated. To confirm that the intermediate lies on the reaction pathway, a control experiment was carried out exactly as above, but in the absence of NAD^+. In this case the precipitate was white, no chromophore being observed between 350 and 500 nm (Figure 1), thus eliminating the possibility of nonspecific labeling of the enzyme by the chemically reactive aldehyde.

Although the chromophore is clearly associated with the active site of the enzyme, the possibility of acyl group transfer, which could lead to an incorrect interpretation of the data, has to be considered. There is no evidence from transient kinetic studies with a rapid-scanning stopped-flow spectrophotometer that the acyl group is transferred during catalysis. This would be revealed by a time-dependent shift in the absorption spectrum of the acyl-enzyme (for example, from sulfur to oxygen). Careful spectroscopic studies have excluded this possibility (Buckley & Dunn, 1982; Dunn & Buckley, 1985). Since the same colored precipitate was isolated, with the same spectroscopic properties, when the reaction mixture was treated with ethanol or denatured with 8 M urea, as well as with perchloric acid, it is unlikely that the perchloric acid treatment is catalyzing acyl group transfer either. It should be noted however that the absorption maximum of the precipitate is shorter than that of the transient (Buckley & Dunn, 1982). This probably reflects the loss of the special active site microenvironment proposed by Buckley and Dunn (1982) on denaturation (irrespective of the denaturating agent).

Isolation of a DACA-Labeled Peptide from the Tryptic Digest. Tryptic digestion of the colored protein precipitate was carried out in an attempt to identify the acylated functional group. After tryptic digestion of the denatured carboxymethylated acyl-enzyme was completed, the 408-nm absorption maximum, characteristic of the chromophore in 0.1 M ammonium bicarbonate, was used to locate the acylated peptide. Unfortunately, the chromophoric label was slowly lost by spontaneous hydrolysis during the tryptic digest and the subsequent isolation procedures. The decay was first order in 30 mM sodium phosphate, pH 8.0, buffer, at 37 °C with a decay constant of 1.8×10^{-5} s^{-1}. Therefore, in order to minimize the time-dependent loss of the chromophore, the tryptic digest was only carried out for 4 h. Nevertheless, considerable loss of the chromophore was experienced during digestion and the following gel filtration. After hydrolysis, the maximum in absorbance of the solutions shifted to 322 nm, characteristic of the free *trans*-4-(*N*,*N*-dimethylamino)-cinnammate ion (Dunn & Buckley, 1985).

Gel filtration of the tryptic peptides obtained from the denatured carboxymethylated acyl-enzyme separated them according to their size, and the absorption spectrum of each fraction was run (Figure 2). The small amount of undigested protein, which eluted from the gel column immediately after the void volume (Figure 2), gave some absorbance at 408 nm, but only one peptide peak (fraction 7) showed a significant absorption maximum at 408 nm, which amounted to about 5% labeling of the peptide present. There was a slight trace of color in the trailing edge of the largest peak (fraction 1) which, since only a partial digest was carried out, could be incompletely digested peptide.

The general profile of the tryptic peptides eluted from the gel column was similar to that obtained by von Bahr-Lindström et al. (1981) (who used 30% acetic acid as elutant). However, the acylated peptide, which eluted at the very end of the run in the present work, was clearly different from the peptide (T20) containing cysteine-302 isolated by those workers, which elutes near the front of the run. The tryptic peptides T13 and T14, which contain Cys-162 and Cys-49, respectively, appear even earlier (supplement 1; Hempel et al., 1984). Of the conserved cysteines only T25, which contains Cys-369, seems

capable of running at the same elution position as the chromophore in Figure 2. T25 has been shown to appear earlier than T5 in the human cytoplasmic enzyme (Hempel et al., 1984) but later in the horse liver isoenzyme (von Bahr-Lindström et al., 1984).

Searching for an Active Site Cysteine by Differential Labeling with [^{14}C]Iodoacetamide. In order to test the possibility that the chromophore was attached to a cysteine as proposed by Hempel et al. (1982), von Bahr-Lindström et al. (1985), and Tu and Weiner (1988), we carried out labeling experiments with a ^{14}C-labeled thiol-modifying reagent. The acylated carboxymethylated protein was isolated as described above by precipitation with perchloric acid, both in the presence of NAD^+ to give active site acylated enzyme and in the absence of NAD^+ to give a control sample of enzyme. Both samples were treated identically in all subsequent experiments. After the carboxymethylation step, the unreacted iodoacetate was removed by gel filtration, and the label was allowed to spontaneously hydrolyze over several hours at pH 8 in 8 M urea, in the presence of a 6.5-fold excess of [^{14}C]-iodoacetamide. The amount of label as measured by the change in absorbance halved (from about 6 to 3 nmol). If a thiol group were released in this process, it would rapidly react with the [^{14}C]iodoacetamide, and about 6000 cpm would be expected to be associated with a peptide labeled in this way. However, there was no significant peak in radioactivity associated with any of the peptides from the trypsin digest and no difference in the small amount of ^{14}C incorporation between the acylated sample and the control. The small amount of label that was incorporated in both the samples and the controls presumably arises either from nonspecific reaction with other groups on the enzyme or from the original precipitate, which was not completely carboxymethylated. The same result was obtained when the label was first removed by the addition of ammonia, before reaction with [^{14}C]iodoacetamide.

From both these experiments there is no evidence for cysteine as the site of attachment of the chromophore.

Primary Sequence of Gel Filtration Fraction 7. Since the spectroscopic characteristics of the chromophore associated with peptide fraction 7 were identical with those of the bulk label in the denatured protein (before tryptic digestion), fraction 7 was further purified for sequence analysis. Fraction 7 was collected and either freeze-dried (to prevent further loss of the label with time if the sample were not to be used immediately) or directly applied to a C18 column equilibrated with 0.1 M ammonium bicarbonate. Figure 3 shows the elution pattern of the peptides contained in fraction 7 after reverse-phase column chromatography. Only one major peak was observed, and only this peak coincided with the chromophore, as shown by the absorbance at 408 nm (in ammonium bicarbonate) although now only 1% of the peptide is labeled. The two minor peaks had no associated color. Fraction 3 was dried under vacuum and sequenced. The first sequencing cycle gave almost exclusively glutamine with a slight trace of glycine. Subsequent steps, however, revealed no trace of contaminating amino acids, only the peptide sequence Gln-Ala-Phe-Gln-Ile-Gly-Ser-Pro-Trp-Arg.

This decapeptide corresponds to T5 in the nomenclature of Hempel et al. (1984). There was some asymmetry of the chromophore in the peak from the reverse-phase column (Figure 3, insert) with the majority of the chromophore being associated with the trailing edge (fraction 3). The fact that only this decapeptide was apparent during sequencing suggests that the asymmetry is due to partial resolution of acylated and nonacylated peptide on the reverse-phase column.

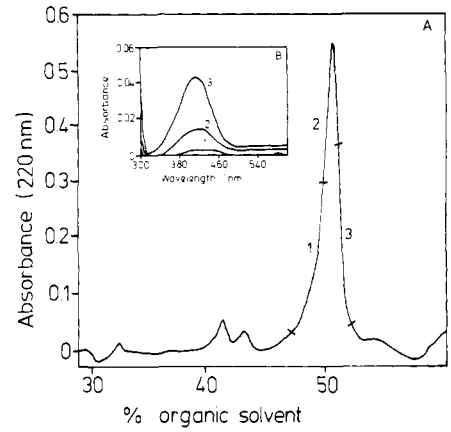


FIGURE 3: (A) Profile at 220 nm obtained from an 8MBC1810μ reverse-phase column when fraction 7 from the gel column (Figure 2) is eluted with a gradient to a solvent mixture of acetonitrile, 2-propanol, and bicarbonate (1:1:1 v/v) as described under Experimental Procedures. (B) Absorbance spectra of all the collected fractions from this column which showed maxima at 408 nm (in 0.1 M ammonium bicarbonate).

Unfortunately, the chromophore was completely lost during the sequencing procedure, so there was no way of confirming which amino acid of peptide T5 was associated with the chromophore. However, on chemical grounds the serine residue is the only likely candidate. Since only about 1% of the sample remained acylated after isolation of the peptide fractions from the C18 column, only a molecular ion with a mass corresponding to the molecular ion of unlabeled peptide T5 was observed (expected = 1189; observed = 1189) by FAB source mass spectrometry. Although only pure decapeptide T5 (Hempel et al., 1984) was found from the sequencing experiment to be associated with the label, our inability to observe a perturbed residue during the sequencing experiments and the inability of mass spectrometry to detect the labeled peptide leave us with no direct evidence that the chromophore is attached to the decapeptide.

Indirect Evidence That the Chromophore Is Attached to Decapeptide T5. The loss of the chromophore on tryptic digestion and during sequencing raises the question of whether the association of the chromophore with T5 might be artifactual. Two possibilities appear to exist.

(1) The chromophore is attached to serine-74, which is therefore an active site residue, but it is progressively lost through hydrolysis during the various stages of the procedure. The oxygen ester that would exist in this case would be susceptible to hydrolysis under basic conditions.

(2) The bulk of the chromophore is attached to some other group on a peptide that is present in only trace amounts.

For possibility 2 to be valid, the labeled peptide present in trace amounts must coelute with the decapeptide, not only on gel filtration but also on reverse-phase HPLC, even though these techniques separate according to fundamentally different properties. Furthermore, in this case digestion of fraction 7 with an enzyme other than trypsin would not be expected to produce peptide fragments in which the serine-containing peptide and the labeled material again coelute.

When fraction 7 (see Figure 2) was redigested with thermolysin before being run on HPLC, the elution pattern shown in Figure 4 was obtained. Although, as would be expected, the profile is more complicated than that shown in Figure 3, color is only incorporated into fractions 5 and 6. On sequencing, both fractions were shown to contain only the C-terminal peptide of T5: Ile-Gly-Ser-Pro-Trp-Arg. Only the unlabeled fragment was detected from mass spectrometry

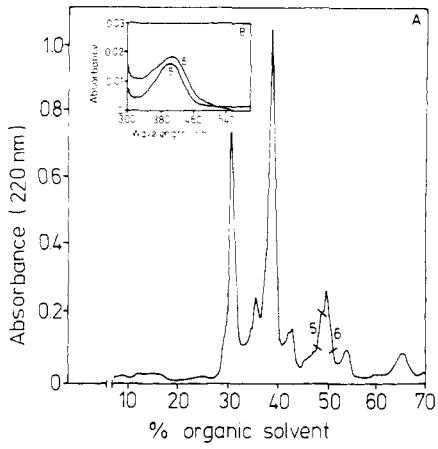


FIGURE 4: (A) Fraction 7 from the gel column (Figure 2) was re-digested with thermolysin as described under Experimental Procedures. This figure shows the profile at 220 nm when this digest was purified on HPLC under conditions identical with those of Figure 3. (B) The absorbance spectra of the fractions that showed a maxima at 408 nm (in 0.1 M ammonium bicarbonate).

(expected = 715; observed = 715).

The close association again of the chromophore with both T5 (Figure 3) and the hexapeptide (Figure 4) favors possibility 1, namely, that the chromophore is covalently attached to the T5 peptide.

The data provide further circumstantial evidence for T5 as the acylated peptide. T5, including the serine residue, is conserved in all mammalian aldehyde dehydrogenase isozymes that have been sequenced (Johansson et al., 1988). It is interesting that a role for serine has also recently been proposed for glyceraldehyde-3-phosphate dehydrogenase (Corbier et al., 1989). Serine-74 is not conserved in the primary sequences of aldehyde dehydrogenase from microorganisms (Kok et al., 1989), but the significance of this is not clear. Of the amino acids on this peptide, only the oxygen on serine-74 could be acylated on chemical grounds. None of the other amino acid residues in the six amino acid peptide isolated after the thermolysin digest of peptide T5 (nor any in the complete 10 amino acid T5 peptide) have side chains suitable to act as nucleophiles to attack the aldehyde to form a hemiacetal, which could allow hydride transfer from the aldehydic carbon to NAD^+.

There are however some difficulties that need explanation. The spectrum of the acyl species after denaturation (λ_{max} = 412 nm in urea) is not characteristic of an ordinary oxygen ester of DACA (λ_{max} of 370 nm; Breaux & Bender, 1976; Dunn & Buckley, 1985). Attempts to resolve this anomaly by acetylation of a synthetic six-residue peptide, acetyl-Ile-Gly-Ser-Pro-Trp-Arg-NH_2, with DACA derivatives failed. A model thioester of *trans*-4-(*N*,*N*-dimethylamino)cinnamic acid and *N*-acetyl-L-cysteine had a λ_{max} of 398 nm in aqueous solution (Buckley & Dunn 1982), which is closer to the 408-nm peak observed for the chromophore in the present work. The thioester was however considerably more stable and showed no loss of absorbance in 12 h in 30 mM sodium phosphate buffer at 37 °C when the acylated carboxymethylated protein lost 31% of its absorbance at 408 nm under the same conditions.

Conclusions

Although aldehyde dehydrogenase is well studied kinetically, relatively little is known about structural aspects of the active site. The tertiary structure remains to be solved, and the known primary structures have provided few clues concerning regions of functional importance (Hempel & Jörnvall, 1987). Reaction of aldehyde dehydrogenase with [^{14}C]iodoacetamide results in the labeling of cysteine-302 (Hempel et al., 1982), which has led to its identification as the active site residue. Preexposure of the enzyme to disulfiram (an inhibitor) reduces the rate of reaction with [^{14}C]iodoacetamide, thus leading to the proposal that the iodoacetamide is modifying a group at or near the active site. This conclusion is supported by the work of von Bahr-Lindström et al. (1985), who found that cysteine-302 is also modified by a coenzyme analogue. Bromoacetophenone, an active site directed reagent that irreversibly inactivates both dehydrogenase and esterase activities, has been shown to modify glutamic acid-268 (Abriola et al., 1987). Finally cysteine-49 has been proposed as an active site residue by Tu and Weiner (1988), on the basis of labeling studies with *N*-ethylmaleimide.

In the present study, the problems of interpretation associated with the use of chemical modifying groups to identify the active site are avoided. By use of a substrate of the enzyme and by trapping an intermediate on the reaction pathway, the chemical probe is clearly covalently attached to a nulceophilic group in the active site. The disadvantage of this method is that, even when the enzyme is denatured, the label is slowly lost by spontaneous hydrolysis in solution. Nevertheless, the evidence suggests that the acyl group is not attached to a cysteine but rather to some other residue, most likely serine-74. This intriguing new proposal should stimulate further interest in the important question of which amino acid residues are fulfilling an essential function at the active site.

Acknowledgments

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Registry No. DACA, 20432-35-3; L-Ser, 56-45-1; aldehyde dehydrogenase, 9028-86-8.

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Evidence for Transition-State Stabilization by Serine-148 in the Catalytic Mechanism of Chloramphenicol Acetyltransferase†,‡

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ABSTRACT: The function of conserved Ser-148 of chloramphenicol acetyltransferase (CAT) has been investigated by site-directed mutagenesis. Modeling studies (P. C. E. Moody and A. G. W. Leslie, unpublished results) suggested that the hydroxyl group of Ser-148 could be involved in transition-state stabilization via a hydrogen bond to the oxyanion of the putative tetrahedral intermediate. Replacement of serine by alanine results in a mutant enzyme (Ala-148 CAT) with k_{cat} reduced 53-fold and only minor changes in K_m values for chloramphenicol and acetyl-CoA. The Ser-148 → Gly substitution gives rise to a mutant enzyme (Gly-148 CAT) with k_{cat} reduced only 10-fold. A water molecule may partially replace the hydrogen-bonding potential of Ser-148 in Gly-148 CAT. The three-dimensional structure of Ala-148 CAT at 2.34-Å resolution is isosteric with that of wild-type CAT with two exceptions: the absence of the Ser-148 hydroxyl group and the loss of one poorly ordered water molecule from the active site region. The results are consistent with a catalytic role for Ser-148 rather than a structural one and support the hypothesis that Ser-148 is involved in transition-state stabilization. Ser-148 has also been replaced with cysteine and asparagine; the Ser-148 → Cys mutation results in a 705-fold decrease in k_{cat} and the Ser-148 → Asn substitution in a 214-fold reduction in k_{cat}. Removing the hydrogen bond donor (Ser-148 → Ala or Gly) is less deleterious than replacing Ser-148 with alternative possible hydrogen bond donors (Ser-148 → Cys or Asn).

Chloramphenicol acetyltransferase (CAT;[1] EC 2.3.1.28) catalyzes the inactivation by O-acetylation of chloramphenicol using acetyl coenzyme A (acetyl-CoA) as acetyl donor. The acetylated antibiotic cannot bind to bacterial ribosomes and consequently is inactive as an inhibitor of protein synthesis [reviewed by Shaw (1983)]. CAT variants have been isolated from both Gram-negative and Gram-positive bacteria where *cat* genes are commonly plasmid-borne (Shaw & Leslie, 1989). The nucleotide sequences of seven *cat* determinants are now known [reviewed by Shaw and Leslie (1989) and Murray et al. (1989)], and the deduced amino acid sequences show a marked degree of homology. It is likely that all CAT variants are trimeric enzymes of identical subunits of M_r 25 000 (Leslie et al., 1986; Harding et al., 1987).

The three-dimensional structure of the catalytically efficient type III variant of CAT (CAT_{III}) has recently been determined (Leslie et al., 1988). The gene encoding this variant (cat_{III}) was originally isolated from a naturally occurring, transmissible plasmid (R387). CAT_{III} is expressed at high levels (30–50% of the total soluble protein) in *Escherichia coli* harboring the cat_{III} gene inserted into appropriate expression vectors (Murray et al., 1988).

The structures of the binary complexes of CAT_{III} with chloramphenicol and CoA have been refined to 1.75 and 2.4 Å, respectively (Leslie et al., 1988; P. C. E. Moody and A. G. W. Leslie, unpublished results). The active site of CAT_{III} is located at the intersubunit interface such that there are three

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‡ Crystallographic coordinates of the Ser-148 → Ala mutant of chloramphenicol acetyltransferase have been submitted to the Brookhaven Protein Data Bank under the name 1CLA coordinates.
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[1] Abbreviations: CAT, chloramphenicol acetyltransferase; TSE buffer, 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl and 0.1 mM EDTA; Tris, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MPD, 2-methyl-2,4-pentanediol; CM, chloramphenicol; CoA, coenzyme A.

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