N-Tosyl-L-phenylalanine Chloromethyl Ketone, a Serine Protease Inhibitor, Identifies Glutamate 398 at the Coenzyme-Binding Site of Human Aldehyde Dehydrogenase. Evidence for a Second "Naked Anion" at the Active Site†

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ABSTRACT: Human aldehyde dehydrogenase isozymes were inactivated by *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), an inhibitor of chymotrypsin. The inactivation was a first-order process that followed saturation kinetics. NAD and chloral when used together protected against inactivation. In steady-state kinetics, TPCK produced only slope effects versus varied NAD, both slope and intercept effects versus varied glycolaldehyde were produced, indicating that TPCK reacted with the same enzyme form with which NAD reacted. Ki values from steady-state kinetics and saturation kinetics were comparable. Use of [^{3}H]-labeled TPCK showed that inactivation was associated with the incorporation of two molecules of TPCK per molecule of enzyme. The label incorporation occurred into a single tryptic peptide and also into a single chymotryptic peptide of the E1 isozyme. Purification of labeled peptides, followed by sequencing, demonstrated that E398 of aldehyde dehydrogenase was labeled. Reaction of a haloketone, TPCK, with a carboxyl group of E398 indicates that E398 occurs as a "naked anion" within the molecule. This paper constitutes identification of the second (after E268) "naked anion" at the active site of aldehyde dehydrogenase.

NAD-linked aldehyde dehydrogenase (EC 1.2.1.3) catalyzes unidirectional irreversible dehydrogenation of aldehydes to carboxylic acids; it also catalyzes the hydrolysis of esters. The dehydrogenation of aldehydes as well as the ester hydrolysis (*1*) proceeds via a covalent acyl-enzyme intermediate (Figure 1). The residue (cysteine 302), involved in the formation of the covalent acyl intermediate, was previously identified via chemical modification (*2*−*5*) and by isolation and characterization of the intermediate with 4-*trans*-(*N*,*N*-dimethylamino) cinnamaldehyde (*6*). The role of cysteine 302 in the formation of the covalent intermediate has been recently confirmed by site-directed mutagenesis (*7*) and by X-ray crystallography of rat tumor inducible aldehyde dehydrogenase (*8*) and bovine mitochondrial aldehyde dehydrogenase (*9*), the latter closely resembling human mitochondrial enzyme. Another amino acid residue, previously identified in our laboratory (*10, 11*) is glutamate 268. The presence of glutamate 268 in the active site as the "naked anion" was established on the basis of its reactivity (*12*) with a haloketone, bromoacetophenone, which also satisfied all criteria for active-site-directed reagents (*10*). Because of its occurrence in a permanently charged form, glutamate 268 was postulated to function as a general base, promoting cysteine 302 ionization (*13*). This role has been also indicated by site-directed mutagenesis (*14*); glutamate 268 localization within the active site pocket has been shown by X-ray crystallography (*8, 9*).

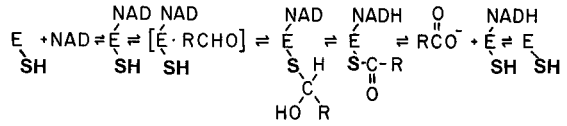

FIGURE 1: Reaction mechanism of aldehyde dehydrogenase. SH or S = cysteine 302; the mechanism is that of the E1 isozyme where NADH dissociation is the slowest step in the reaction sequence.

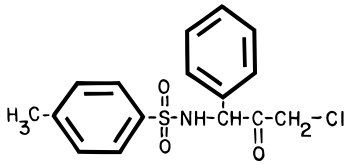

FIGURE 2: Chemical structure of *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK).

In its reaction involving ester hydrolysis, aldehyde dehydrogenase resembles cysteine and serine proteases. Cysteine proteases also show some structural resemblance to aldehyde dehydrogenases (*15*). It has been observed in this laboratory that a variety of serine protease inhibitors inhibit aldehyde dehydrogenase; these include bromoacetophenone (*10*), phenylmethyl sulfonyl fluoride, and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK). TPCK was originally designed as a specific site-directed inhibitor of chymotrypsin (*16*) and is still commonly used to remove chymotrypsin from preparations of trypsin. The structure of TPCK is shown in Figure 2.

In recent years, many amino acid sequences have been obtained for a variety of aldehyde dehydrogenases from gene cloning. Linear alignment of these sequences was first

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attempted in 1993 by Hempel et al. (*17*), in an effort to locate totally conserved residues. Twenty-three totally conserved residues were thus identified, which in addition to 11 glycines and 3 prolines, included R84, K192, C302, T384, E399, F401, N421, N454, and S471. Although, except for cysteine 302, none of the above residues were found to directly participate in aldehyde dehydrogenase catalysis, some (lysine 192 and glutamate 399), when mutagenized (*18, 19*), have been found to affect catalytic activity. In X-ray crystallography both were found in the active site pocket (*8, 9*) and were postulated to form hydrogen bonds with the coenzyme (*9*). Glutamate 399, localized in the proximity of nicotinamide ribose (*9*), was claimed to form two hydrogen bonds with ribose.

In this paper, we characterize the reaction of glutamate 398 with TPCK and demonstrate that glutamate 398 occurs in the aldehyde dehydrogenase molecule as a "naked anion". Two isozymes (E1, cytoplasmic, and E2, mitochondrial) of human NAD-linked aldehyde dehydrogenase which were originally purified in our laboratory to homogeneity (*20*), have been used during this investigation.

MATERIALS AND METHODS

Materials. NAD was from Boehringer Mannheim. 2-Mercaptoethanol, trifluoroacetic acid (TFA), glycolaldehyde, *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), *N*-tosyl-L-lysyl chloromethyl ketone (TLCK), and TLCK-treated chymotrypsin were from Sigma Chemical Co. [^{3}H]-Labeled TPCK was obtained by tritium exchange performed by Du Pont NEN custom labeling service. Methanol (HPLC grade) was from Fisher Scientific. TPCK-treated trypsin was from Worthington. Propionaldehyde was from Aldrich; it was redistilled before use. Aldehyde dehydrogenases, E1 and E2 isozymes, were purified from postmortem human liver (*20*); purity was verified by starch gel electrophoresis and by specific activity determinations. Prior to use, the enzymes were exhaustively dialyzed to remove 2-mercaptoethanol, used to protect the enzymes during storage. As a result of the sensitivity of both enzymes to air oxidation, nitrogen-saturated buffers were used throughout.

Enzyme Assay and Steady-State Kinetics. Dehydrogenase activity was assayed in 0.1 M pyrophosphate buffer, pH 9.0, containing 1 mM EDTA, 0.5 mM NAD, and 1 mM propionaldehyde, at 25 °C in cuvettes of 1 cm light path. An extinction coefficient of 6.22 mM^{-1} cm^{-1} for NADH was used for calculation of reaction rates. Steady-state kinetics was done at 25 °C in 30 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA, at different inhibitor concentrations employing varied glycolaldehyde or varied NAD. Kinetic parameters were obtained using the HYPER program of Cleland (*21*), employing the nonlinear regression fit of rates versus substrate concentration.

Protein Determination. Protein was determined by 280 nm absorption by employing an extinction coefficient of 0.96 for E1 and 1.05 for E2 for a 1 mg/mL solution at 1 cm light path (*20*). Lowry et al. (*22*) and Goa (*23*) procedures were also employed.

Incubation with TPCK and Saturation Kinetics. Enzyme inactivation was studied by preincubating the enzymes with inhibitors in 30 mM sodium phosphate, pH 7.0, containing 1 mM EDTA. All incubations were started by addition of TPCK. TPCK was dissolved in ethanol, the final enzyme/inhibitor incubation mixture contained up to 5% v/v ethanol. In substrate protection experiments, substrates (expected to protect) were added and mixed with the enzymes before inhibitors were added. After different time periods, aliquots were removed for assay of activity remaining. Saturation kinetics was done as described by Kitz and Wilson (*24*). The results obtained were plotted as the log of the % activity remaining versus time at various concentrations of the inhibitor. The reciprocals of $k_{apparent}$, obtained from the primary plots, were then plotted versus reciprocals of inhibitor concentration to obtain dissociation constants, Ki, and rates of covalent bond formation, k3.

Stoichiometry of Label Incorporation. The E1 isozyme was incubated with unlabeled TPCK in 30 mM phosphate buffer, pH 7.0, containing 1 mM EDTA. After 30 min of incubation at 25 °C, aliquots were withdrawn and assayed for enzyme activity. Following incubation of E1 isozyme with 1−5 equiv of [^{3}H]-labeled TPCK and exhaustive dialysis, stoichiometry was also determined by counting the radioactivity of the protein solution. The E1 isozyme was also titrated directly with TPCK, and the titration was monitored by determination of the activity remaining.

Chemical Modification of E1 Isozyme with TPCK. Prior to modification, the enzyme was dialyzed exhaustively (24 h, 4 °C) against several changes of 30 mM sodium phosphate, pH 7.0, containing 1 mM EDTA, to remove NAD and 2-mercaptoethanol. Modification of the E1 isozyme was done by incubating the enzyme with a 2-fold molar excess of [^{3}H]-labeled TPCK in 30 mM phosphate buffer, pH 7.0, containing 1 mM EDTA. The reaction was allowed to proceed for about 30 min on ice and was monitored at selected time intervals by the removal of an aliquot to determine the activity remaining. After modification, the protein was dialyzed, reduced, alkylated, and subjected to tryptic and chymotryptic digestion as previously described (*3*).

Peptide Mapping and Purification of the Labeled Peptides. The complete tryptic digest was chromatographed with a linear gradient from 0.1% v/v aqueous TFA to 100% methanol on a Supelco C-8 RP-HPLC column. The chymotryptic map was obtained using a Waters C-18 RP-HPLC column with a mobile phase of 0.1% v/v TFA in water, and the column was developed in a linear 0−100% methanol gradient. Throughout purification, 1.0 mL/min fractions were collected and aliquots were removed for scintillation counting.

Isolation and Analysis of the Labeled Peptides. Further purification of peptides, containing the radioactive label (fractions 39−41 and 32−33), was carried out by employing a four-step procedure. The first step involved chromatography on Waters μ Bondapak C-18 (tryptic digest) and Supelco C-8 (chymotryptic digest) reverse-phase columns with a linear gradient of methanol in 0.1% TFA. The next step involved chromatography on a Supelco DP-3LC reverse-phase column in the same gradient system. This was followed by rechromatography on the same column with a much shallower gradient in the range of 30−40% of methanol. Finally, a Supelco C-4 reverse-phase HPLC column was used with a linear gradient of methanol in 0.1% TFA. The fractions collected after each purification step

Table 1: Protection of the E1 Isozyme by NAD and Chloral against TPCK Inactivation[a]

TPCK (μM)	protecting agent	% activity remaining
20	none	13
20	NAD (1.5 mM)	28
20	NAD (1.5 mM) + Chloral (1 mM)	89
0	none	100

[a] Activity remaining was determined after 5 min preincubation at 25 °C of the E1 isozyme (4.6 μM), with and without inhibitor and/or protecting agents, in 30 mM sodium phosphate buffer, 1 mM EDTA, pH 7.0. After 5 min, the activity in 20 μL aliquots (at 1/150 dilution) was determined by employing a standard assay system. When chloral was used as a protecting agent, activity was determined versus a control that contained the same amount of chloral.

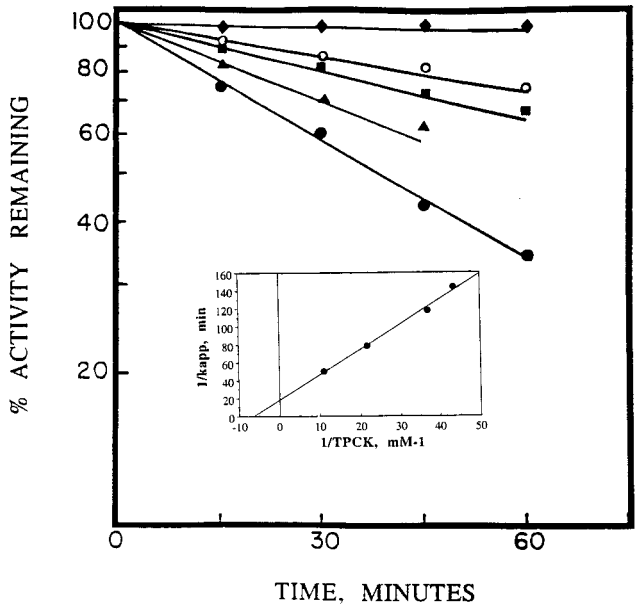

FIGURE 3: Saturation kinetics with the E2 isozyme. The solution of the E2 isozyme (3.6 μM) in 30 mM sodium phosphate buffer, 1 mM EDTA, pH 7.0 contained TPCK in the amount of 0 μM (◆), 23 μM (○), 27 μM (■), 46 μM (▲), and 91 μM (●). Incubation was carried out at 25 °C; aliquots were withdrawn at various times and assayed for activity remaining. Inset shows a double reciprocal plot of $1/K_{app}$ vs 1/[TPCK]. Ki_{TPCK} calculated from this experiment was 140 μM.

were checked for radioactivity by employing an LKB 1219 RACBETA scintillation counter. Sequence analysis of tryptic peptides was done by W. M. Keck Foundation Biotechnology Resources Laboratory, New Haven, CT, who supplied us with material for scintillation counting. Sequence analysis of the chymotryptic peptide and scintillation counting was done by M. Dryjanski employing a model 6600/6625 MiliGen/Biosearch sequencer and an LKB 1219 RACBETA scintillation counter at Wroclaw University, Poland.

RESULTS

Inactivation of Aldehyde Dehydrogenase. Incubation of either E1 or E2 isozyme with TPCK at pH 7.0 led to a time-dependent activity loss. The activity could not be regained on dilution. With the E1 isozyme, the activity loss was extremely rapid and the time course of inactivation at 1 and 2 molar equiv of TPCK was determined by preincubation on ice. The inactivation of the E2 isozyme by TPCK was considerably slower; also, inactivation was incomplete. The inactivation of both isozymes of aldehyde dehydrogenase occurred much more rapidly than that with chymotrypsin, employed as control. The activity of the enzyme inhibited by TPCK could not be regained by exhaustive dialysis or by reduction with 2-mercaptoethanol. TLCK, a trypsin inhibitor, also inactivated E2 isozyme, but much more slowly than did TPCK.

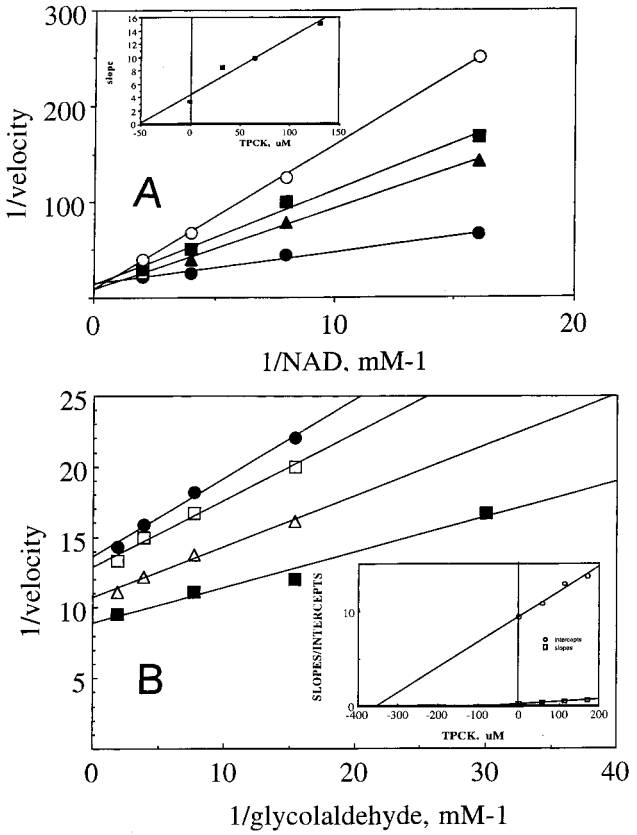

FIGURE 4: Steady-state kinetics with the E2 isozyme with varied NAD and glycolaldehyde. (A) Steady-state kinetics with varied NAD. [Glycolaldehyde] = 156 μM; [TPCK] = 0 μM (●), 40 μM (▲), 80 μM (■), and 120 μM (○). Inset shows a secondary plot of slopes vs TPCK concentration. (B) Steady-state kinetics with varied glycolaldehyde. [NAD] = 500 μM; [TPCK] = 0 μM (■), 57 μM (△), 113 μM (□), and 170 μM (●). Inset shows replot of slopes (■) and intercepts (○) vs TPCK concentration.

Substrate Protection. The inactivation of both E1 and E2 isozymes by TPCK was protected by NAD + chloral; NAD alone also protected but to a lesser extent (Table 1). NAD (1.5 mM) is saturating for the E1 isozyme whose Km for NAD is 10 μM (*25*).

Reaction Specificity from Saturation Kinetics. The activity loss of the E2 isozyme was a first-order process that followed saturation kinetics (Figure 3). The secondary plot was linear with a pronounced *y*-axis intercept. The Ki value for TPCK from saturation kinetics (96 and 140 μM from two experiments) and the first-order rate constant of the covalent bond formation (calculated from the *y*-axis intercept of secondary plot) was 2.4 min^{-1}.

Recognition Specificity from Steady-State Kinetics. When employed as an inhibitor in steady-state kinetics (Figure 4), TPCK gave a competitive pattern versus varied NAD (Figure 4A) and a noncompetitive pattern versus varied glycolaldehyde (Figure 4B). This pattern suggests that TPCK reacts with the enzyme form that reacts with NAD. The Ki values

Table 2: Kinetic Characterization of TPCK as an Inhibitor of Human Aldehyde Dehydrogenase E2 Isozyme[a]

varied substrate (range, μM)	fixed substrate (μM)	inhibition form	Ki (slope) (μM)	Ki (intercept) (μM)
NAD (60−500)	glycolaldehyde (132)	C	50	
NAD (60−500)	glycolaldehyde (156)	C	55	
glycolaldehyde (60−503)	NAD (500)	NC	95	375
glycolaldehyde (30−523)	NAD (500)	NC	150	302

[a] C = competitive; NC = noncompetitive. For a compulsory ordered mechanism and competitive inhibition, Ki (slope) = Ki (TPCK); for noncompetitive inhibition, Ki (slope) = Ki (TPCK) (1 + [NAD]/Ki (NAD) and Ki (intercept = Ki (TPCK) (1+ [NAD]/Km (NAD).

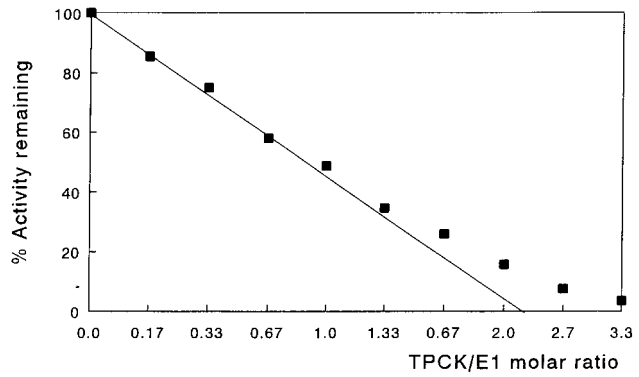

FIGURE 5: Titration of the E1 isozyme with TPCK. E1 isozyme (3.06 μM) was incubated with TPCK (0−10 μM) in 30 mM phosphate buffer, pH 7.0, 1 mM EDTA at 25 °C. After a 30 min incubation, aliquots were withdrawn and assayed for enzyme activity.

obtained from steady-state kinetics versus varied NAD and glycolaldehyde are listed in Table 2.

Stoichiometry of Incorporation of TPCK. Both E1 and E2 isozymes retained label from TPCK following dialysis. The stoichiometry of TPCK incorporation of one molecule of TPCK per molecule of E1 isozyme was obtained by determination of the radioactivity of tritium-labeled TPCK following exhaustive dialysis after inactivation. When determined directly by titration with TPCK, which interacts with the E1 isozyme instantaneously (Figure 5), the stoichiometry was two molecules of TPCK per mole of E1 isozyme.

Specificity of Incorporation of TPCK. The E1 isozyme was modified with [^{3}H]-labeled TPCK and, following enzymic digestion, subjected to peptide mapping (Figure 6). Tryptic (Figure 6A) and chymotryptic (Figure 6B) maps each showed only one labeled peptide.

Analysis of Labeled Peptide from Tryptic Digest. The labeled peptides were purified to apparent homogeneity. The radioactive label (700 cpm) appeared in the first sequencing cycle. The second cycle contained 32 cpm with no counts detected in the following cycles. However, N-terminal sequencing of the peptide from tryptic digest revealed the presence of two peptides with a ratio of primary to secondary sequence of about 4:1. Amino acid sequence analysis of the peptides allowed for identification of the primary component as peptide 398−409 and the secondary component as peptide 420−434 of the E1 isozyme (Table 3). Approximately 50% of each sequencing cycle fraction was counted on a scintillation counter to determine which cycles contained the label. The radioactivity was found only in the first cycle of Edman degradation. On the basis of the

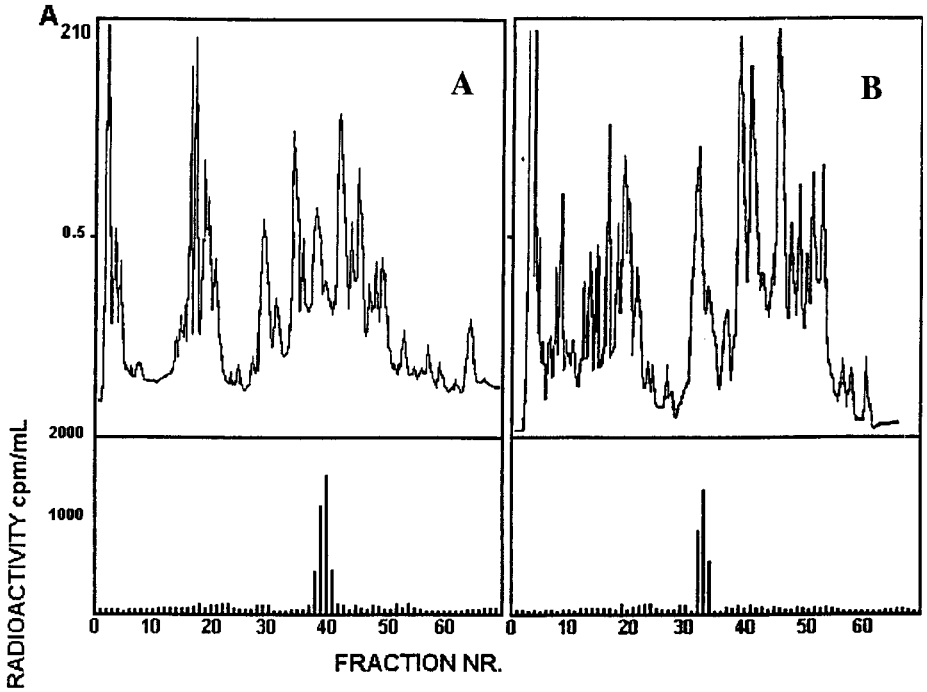

FIGURE 6: HPLC profiles of peptides generated by tryptic and chymotryptic digestion of [^{3}H]-TPCK-labeled E1 isozyme. (A) HPLC profile after tryptic digestion. The column was a Supelco C-8 reverse phase. (B) HPLC profile of peptides generated upon chymotryptic digestion. The column used was a Waters C-18 reverse phase. A linear gradient of methanol in 0.1% v/v TFA was employed at a flow rate of 1 mL/min. The [3H] content of the 1 mL fractions collected was determined by liquid scintillation counting.

Table 3: Peptides Purified Following Labeling of E1 Isozyme with TPCK and Label Identification

digestion	sequence of peptides purified	position in E1 isozyme sequence	labeled residue
trypsin	EEIFGPVQQIMK	398−409	E398
	ANNTFYGLSAGVFYK	420−434	
chymotrypsin	SNVTDEMRIAKEEIF	387−401	E398

stoichiometry of inactivation (TPCK/E1 isozyme molar ratio of 2:1), specific activity of [^{3}H]-TPCK used for enzyme modification (22 000 cpm/nmol), and the initial yield of primary and secondary sequences (58.2 for 398−409 peptide and 16.2 for 420−434 peptide), the level of radioactivity found in the first cycle of sequencing appeared to be more in line with the 398−409 peptide, suggesting that E398 was labeled.

Analysis of Labeled Peptide from Chymotryptic Digest. Because the result from tryptic digest showed label localization in the first cycle of sequencing and the presence of two peptides, making absolute label assignment difficult, chymotryptic digest of the [^{3}H]-TPCK-labeled E1 isozyme was subjected to peptide mapping in order to obtain more satisfactory results. N-terminal sequence analysis of the peptide containing radioactive label (fractions 32−33, Figure 6B) revealed the presence of only one peptide (residues 387−401 of the primary structure of the E1 isozyme). The radioactive label was found in the 12th cycle of sequencing (520 cpm/32 pmol); the label was found in E398. Cycles 1−11 contained no label; cycle 12 contained 520 cpm; cycle 13 contained 26 cpm, with no detectable label in the following cycles.

DISCUSSION

TPCK has been found during this investigation to be a potent inhibitor of aldehyde dehydrogenase, more effective with the cytoplasmic E1 isozyme than the mitochondrial E2 isozyme. The inactivation of the cytoplasmic E1 isozyme occurred so rapidly at stoichiometric amounts of TPCK that the enzyme could be conveniently titrated by TPCK (see Figure 5). Because of the fast inactivation, the E1 isozyme could not be used for kinetic experiments necessary for inhibitor characterization. Thus, all kinetic experiments were done on the E2 isozyme. Saturation kinetics demonstrated that the inactivation of E2 isozyme by TPCK involved a covalent bond formation. There was no activity regained upon dilution or prolonged dialysis. Prior to covalent bond formation, TPCK was specifically recognized by the enzyme as demonstrated by the presence of *y*-axis intercepts in secondary plots from saturation kinetics. There was a substrate protection against inactivation by TPCK, which was most pronounced when NAD and chloral (an aldehyde-competitive inhibitor) were used together. Total (or almost total) inactivation of the E1 isozyme was obtained. However, inactivation of the E2 isozyme was not total but proceeded to ca. 20% activity remaining. It closely resembled our previous experiments of chemical modification with iodoacetamide (*2*), where although the catalytic cysteine 302 residue was modified (*3*), the inactivation proceeded to 20% activity remaining and activity could not be totally abolished. Resistance of the E2 isozyme to chemical modification remains unexplained, and the reasons are not apparent from X-ray crystallography (*9*).

With inhibitors that form covalent bonds, specific recognition is frequently considerably faster than the covalent bond formation, allowing these inhibitors to be studied by steady-state kinetics. Such a study with TPCK (Figure 3) allowed us to delineate the site of its interaction with aldehyde dehydrogenase. TPCK is a competitive inhibitor versus NAD and a noncompetitive inhibitor versus aldehyde, showing that TPCK reacted with the same enzyme form with which NAD reacted. Ki values for TPCK could be determined directly from steady-state kinetics with varied NAD (Table 2). Ki_{slope} and $Ki_{intercept}$ values, derived from noncompetitive primary plots with varied glycolaldehyde, when adjusted for the effect of constant NAD (see footnotes to Table 2), were found to resemble directly determined Ki_{TPCK} values. The reason for the Ki_{TPCK} from saturation kinetics being 2−3 times larger than that from steady-state kinetics is unknown. Thus, it appeared that TPCK reacted with aldehyde dehydrogenase at the coenzyme-binding site.

Two equivalents of TPCK were needed to abolish catalytic activity of the E1 isozyme (see Figure 5). This stoichiometry corresponds to the active site number determined by NADH titration (*26*). Further experiments employing labeling with [^{3}H]-TPCK demonstrated conclusively specificity of label incorporation from [^{3}H]-TPCK and identified E398 as the amino acid residue of aldehyde dehydrogenase that incorporated the label. There was no other labeled amino acid residue that could be identified. Thus, TPCK fulfilled all criteria previously delineated (*16*) to characterize affinity reagents.

The affinity reagents are bifunctional and contain both the recognition part of the molecule and the reactive part. In TPCK, the toluenesulfonyl-L-phenylalanine part of the molecule constitutes the recognition site, while the chloromethyl ketone part is the reactive part of the molecule. TPCK has been designed (*16*) as an inhibitor for chymotrypsin to resemble chymotrypsin substrates in structure; a separate reagent, TLCK, was designed as a trypsin inhibitor. TPCK reacts with histidine 57 of chymotrypsin, a member of the catalytic triad involved in charge transfer. Serine and cysteine proteases are strongly inhibited by aldehydes and resemble aldehyde dehydrogenase in the hemiacetal structure of their transition state (see Figure 1) (*27, 28*). Peptide aldehydes are well-recognized transition-state analogues of serine and cysteine proteases. It is not clear, however, why aldehyde dehydrogenase recognizes both TPCK and TLCK, with pronounced preference for TPCK. Possibly, because both compounds resemble in structure aldehyde dehydrogenase substrates, some of which are bulky and apolar. In fact, TPCK is a better inhibitor of aldehyde dehydrogenase than of chymotrypsin. The Ki value of the mitochondrial E2 isozyme for TPCK is 6 times smaller than that for chymotrypsin (50 μM vs 300 μM; see ref *29*); that for the cytoplasmic E1 isozyme is probably even smaller but was impossible to determine due to the rapidity of inactivation. With aldehyde dehydrogenase, however, TPCK is a competitive inhibitor versus NAD, which does not bear any obvious structural resemblance to chymotrypsin substrates. Possibly TPCK binds at the aldehyde substrate binding site with the reactive group orientation overlapping with the coenzyme. Our experiments show that substrate protection by chloral in the presence of saturating NAD (Table 1) is far superior to that by NAD alone, supporting this conclusion.

Haloketones have been frequently incorporated into molecules designed as affinity reagents. Haloketones are reactive and are capable of reacting with any enzyme nucleophile that occurs close to haloketone group, after binding to the enzyme molecule. These reactions with sulfhydryl, hydroxyl, and amino groups of various amino acid residues of enzymes are well recognized (*30*). Carboxyl groups are normally unreactive, as carboxylate anions in aqueous solvents are poor nucleophiles. In organic solvents, carboxyl groups are not charged. Uncharged carboxyl groups are even less reactive; haloketones are usually synthesized in anhydrous acetic acid as a solvent. Only in certain conditions are haloketones also capable of reacting with carboxyl groups of proteins. Such a reaction has been demonstrated to occur with an aspartyl carboxyl, located at the active site of pepsin (*31*), and *p*-bromophenacyl bromide, with formation of an ester derivative. Such reactions also occur in model systems in organic solvents, in the presence of crown ether catalysts (*12, 32, 33*), which orient the molecules. In the presence of crown ethers (*12, 33*), the carboxyl groups are fully charged and occur as "naked anions" and in this form readily react with haloketones. Thus, reactivity of glutamate 398 with TPCK shows that glutamate 398 occurs in aldehyde dehydrogenase molecules as a "naked anion".

Recent results from X-ray crystallography demonstrate that E399 is a part of the catalytic domain and is localized in the active site pocket of aldehyde dehydrogenase (*8, 9*). Liu et al. (*8*) state that E333 (E399 equivalent) is located in the stem of the catalytic funnel between the catalytic thiol and the nicotinamide C4. Steinmetz et al. (*9*) describe E399 residue that is in proximity to the nicotinamide ring of NAD, which participates in the alignment of the coenzyme. E398, that has been shown during this investigation to react with TPCK, was never previously identified as important to catalysis. Chemical modification with TPCK, described in this paper, demonstrates that TPCK reacts with E398 before NAD in sequence, since TPCK is competitive with NAD. This reactivity shows that E398 is positioned within the molecule in an extremely hydrophobic environment and that its "naked anion" state is independent of coenzyme and probably substrate binding. Since TPCK fulfills all criteria for an affinity reagent, with which E398 shows unusual reactivity, importance of E398 to aldehyde dehydrogenase catalysis is suggested by the results presented in this paper.

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