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Articles

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

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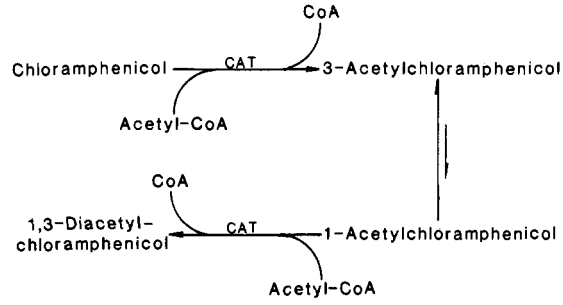
ABSTRACT: Bacterial resistance to the antibiotic chloramphenicol is normally mediated by chloramphenicol acetyltransferase (CAT), which utilizes acetyl coenzyme A as the acyl donor in the inactivation reaction. 3-(Bromoacetyl)chloramphenicol, an analogue of the acetylated product of the forward reaction 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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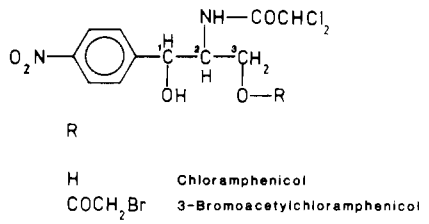


FIGURE 1: Structure of the antibiotic chloramphenicol and the bromoacylated derivative 3-(bromoacetyl)chloramphenicol.

has been cloned and its nucleotide sequence established (I. Murray, A. Hawkins, and W. V. Shaw in preparation). The primary structures of the type I and type III variants are identical at 46% of the amino acid residues. Acetyl-transfer enzymes have previously been found to proceed by either sequential [see choline *O*-acetyltransferase (Hersh, 1982) and kanamycin *N*-acetyltransferase (Radika & Northrop, 1984)] or double-displacement mechanisms [see arylamine *N*-acetyltransferase (Riddle & Jencks, 1971)]. The bimolecular reaction catalyzed by CAT proceeds by a ternary complex (sequential) mechanism (Tanaka et al., 1974; Kleanthous & Shaw, 1984) with no evidence for the involvement of an acyl-enzyme intermediate (Zaidenzaig & Shaw, 1978). Although the substrates in both the forward and reverse reactions of CAT bind independently of each other, a preference for acetyl-CoA binding first in the forward reaction can be demonstrated (Kleanthous & Shaw, 1984).

The identification of amino acids at or near the catalytic center of CAT variants has so far been limited to the use of nonspecific inhibitors and thiol-specific reagents (Zaidenzaig & Shaw, 1978). Such reagents have detected critically disposed cysteinyl and histidyl residues, the modification of which abolishes acetyltransferase activity (Zaidenzaig & Shaw, 1978; Fitton & Shaw, 1979).

We report here the synthesis, characterization of inhibition, and site of modification of CAT by 3-(bromoacetyl)chloramphenicol, an extremely potent active site directed inhibitor. The role of a uniquely reactive histidyl residue involved in the covalent modification and inactivation of CAT is discussed.

Experimental Procedures

Materials

Orthophosphoric acid and sodium perchlorate were obtained from BDH. 2-Propanol was obtained from Koch-Light Limited.

Methods

Preparation of 3-(Bromoacetyl)chloramphenicol. Two methods were developed for the synthesis of 3-(bromoacetyl)chloramphenicol.

Method A. Typically, chloramphenicol (1 g, 3.1 mmol) was suspended in anhydrous acetonitrile (10 mL; distilled from CaH_2) at 0 °C, and bromoacetyl bromide (0.27 mL, 3.1 mmol) was added followed by dry pyridine (0.27 mL, 3.4 mmol). After 30 min at 0 °C, the mixture was stirred for a further 30 min at room temperature. The reaction was monitored by TLC on silica gel plates (Kieselgel 60 F_{254}, Merck), eluting with chloroform/methanol (19:1 v/v). 3-(Bromoacetyl)-chloramphenicol was separated from chloramphenicol, 1-(bromoacetyl)chloramphenicol, and 1,3-bis(bromoacetyl)-chloramphenicol by "flash" column chromatography (Kieselgel 60 PF_{254} Merck), as described by Kleanthous & Shaw (1984). Fractions containing 3-(bromoacetyl)chloramphenicol were identified by TLC and evaporated to give a white crystalline solid (0.73 g, 52% yield). Recrystallization from ethyl acetate/hexane gave white prisms: mp 94–96 °C; ^1H NMR ($[^2H_6]Me_2SO$) δ 4.06 (2 H, s), 4.23 (3 H, m), 5.00 (1 H, m), 6.2 (1 H, d, exchanges with D_2O), 6.39 (1 H, s), 7.58 (2 H, d), 8.14 (2 H, d), and 8.48 (1 H, br s, slowly exchanges with D_2O). Anal. Calcd for $C_{13}H_{13}BrCl_2N_2O_6$: C, 35.16; H, 2.95; N, 6.31. Found: C, 35.21; H, 2.94; H, 6.32.

Method B. Bromoacetic acid (0.46 g, 3.3 mmol) and *N*,*N*′-dicyclohexylcarbodiimide (0.92 g, 4.4 mmol) were dissolved in dry acetonitrile (10 mL) containing pyridine (0.36 mL, 4.4 mmol). To the stirred mixture was added chloramphenicol (1 g, 3.1 mmol), and the reaction stirred at room temperature for 1 h. The precipitated *N*,*N*′-dicyclohexylurea was removed by filtration and the product isolated by column chromatography and characterized as described under Method A.

3-(Bromoacetyl)[^{14}C]chloramphenicol. 3-(Bromoacetyl)[^{14}C]chloramphenicol [D-(−)-*threo*-*N*-(2,2-dichloro-[1,2-$^{14}C_2$]acetyl)-2-amino-1-(*p*-nitrophenyl)-1,3-propanediol 3-(bromoacetate)] was prepared with method A by the addition of [^{14}C]chloramphenicol (New England Nuclear; sp act. 43.2 mCi/mmol) to the reaction mixture. The product was isolated with a specific activity of 0.12 μCi/μmol.

3-(Bromo[^{14}C]acetyl)chloramphenicol. 3-(Bromo[^{14}C]-acetyl)chloramphenicol was prepared by method B with bromo[1-^{14}C]acetic acid (Amersham International; sp act. 58 mCi/mmol). The product was isolated, as described above, with a specific activity of 0.45 μCi/μmol.

Inhibition of Acetylation and Hydrolytic Activities of CAT by 3-(Bromoacetyl)chloramphenicol. CAT (1.6 mg, 64 nmol), after exhaustive dialysis against TSE buffer (pH 7.5) to remove bound chloramphenicol, was incubated with a 1.5-fold excess of 3-(bromoacetyl)chloramphenicol (90 nmol) in a final volume of 110 μL for 2 min at 25 °C. The analogue was dissolved in acetonitrile, the final concentration of which was 1.9 M in each incubation. Control experiments indicated that the enzyme remained fully active, when incubated under the same conditions, in the presence of concentrations of acetonitrile as high as 3.2 M. Aliquots (10 μL) were then assayed for residual acetylation and hydrolytic activities by the spectrophotometric assays described by Kleanthous & Shaw (1984), which are based on the absorbance at 412 nm of the 2-nitro-5-thiobenzoate dianion released as a result of the coupling of the product, reduced CoA, with 5,5′-dithiobis(2-nitrobenzoic acid).

Inactivation of CAT by 3-(Bromoacetyl)chloramphenicol in the Absence and Presence of Chloramphenicol. The time course of chemical modification was routinely measured at pH 7.5 in TSE buffer at 25 °C. Each incubation (1 mL) contained CAT (10 pmol), 3-$BrCH_2COCm$ (57–245 pmol), and acetonitrile (at a final concentration of 38 mM). Experiments intended to show protection also contained chloramphenicol (30 nmol). At appropriate time intervals, 40-μL aliquots were assayed for residual CAT acetylation activity as described above.

Incorporation of Label after Inactivation of CAT by 3-(Bromo[^{14}C]acetyl)chloramphenicol. CAT (0.8 mg, 32 nmol), in TSE buffer, was incubated in a final volume of 60 μL with a range of 3-$BrCH_2$*COCm concentrations (5–35 nmol, 2.2–15 nCi) in a final acetonitrile concentration of 3.2 M for 2 min at 25 °C. The residual CAT activity at each concentration of inhibitor did not alter on increasing the time of incubation. Aliquots (10 μL) were diluted into TSE buffer containing 0.1 M 2-mercaptoethanol and then into TSE buffer alone prior to assay for residual enzymic activity. Covalent incorporation was monitored by applying 20-μL aliquots to 2-cm squares of Whatman 3MM filter paper, precipitating

protein-bound counts with ice-cold TCA, and washing off excess reagent as described by Fraij & Hartman (1983). When the filter squares were counted, corrections were made for quenching by the paper and the protein applied to it.

Hydrolysis of Labeled Protein and Thin-Layer Electrophoresis of Modified Amino Acids. CAT (5 mg, 0.2 μmol) in TSE buffer, (1 mL) was inactivated with 3-$BrCH_2$*COCm (0.33 μmol, 0.15 μCi) as described above. The excess reagent was removed by repeated dialysis against water and one-tenth (20 nmol) of the resulting protein suspension was acid hydrolyzed, in vacuo in HCl (6 M) for 24 h at 110 °C. The hydrolysate was dried over NaOH and redissolved in 200 μL of H_2O from which two equivalent samples (50 μL, 5 nmol) were spotted onto a 20 × 20 cm TLC plate (Polygram Sil G silica). Electrophoresis was carried out in a Shandon flat-bed electrophoresis tank at pH 6.5 for 2 h at 15–18 mA with neutral, basic, and carboxymethylated amino acid standards (25 nmol). The plate was stained with fluorescamine, and one of the two [^{14}C]carboxymethylated hydrolysate tracks was cut into 1 × 3 cm strips and counted (no corrections were made for quenching) while the remainder of the plate was autoradiographed.

(Carboxymethyl)histidine derivatives were prepared by the reaction of histidine, in ammonium bicarbonate (0.05 M, pH 8.5), with a 5-fold excess of iodoacetic acid at room temperature and with stirring for 48 h. During this time, a pH of 8 or greater was maintained. The mixture was subjected to electrophoresis at pH 6.5 on Whatman 3MM paper at 3 kV for 40 min and revealed, after fluorescamine staining, four spots including that corresponding to histidine. Each product was eluted from the paper with 5% NH_3 in H_2O. Samples were dried and redissolved in H_2O prior to identification by their relative electrophoretic mobilities at pH 6.5 (Adamson et al., 1984).

Cleavage of Ester Linkage between Chloramphenicol and Carboxymethyl-CAT, Tryptic Digestion, and Peptide Mapping. Two identical samples of CAT (9 mg, 0.36 μmol) were separately modified with a 1.5-fold excess of 3-$BrCH_2CO$*Cm (0.50 μmol, 0.062 μCi) or 3-$BrCH_2$*COCm (0.52 μmol, 0.24 μCi) in a final volume of 1 mL of TSE, as described above. A third sample of CAT was not modified but was subsequently manipulated in an identical fashion. All three samples were incubated overnight at 40 °C, dialyzed against water, and lyophilized. In the first case, 93% of the ester-linked [^{14}C]-chloramphenicol was lost on dialysis in marked contrast to the [^{14}C]carboxymethyl moiety, all of which remained attached to the protein. All three samples were then denatured, reduced, and *S*-carboxymethylated (by cold iodoacetate) essentially as described by Zaidenzaig & Shaw (1978), dialyzed against water, and lyophilized. The precipitates were resuspended in 0.05 M NH_4HCO_3 (pH 8.5) and digested with trypsin at 37 °C, as described by Packman & Shaw (1981). The digestions were stopped after 4 h by the addition of acetic acid (20% v/v). The peptides were then centrifuged to remove insoluble material and separated by reverse-phase high-pressure liquid chromatography using a C_{18}-radial compression cartridge (Z-module, Waters) and a gradient system of 100% A (0.1% H_3PO_4 containing 0.01 M $NaCl_4$) to 75% B (A + 60% 2-propanol) (Wilson et al., 1981) in 45 min with a flow rate of 1.5 mL/min. The injection volume varied from 0.02 mL (1 nmol) for analytical runs to 0.90 mL (45 nmol) for preparative runs. Peptides were detected by absorbance at 220 nm and collected manually. Each sample was desalted by Sep-pak (Waters) prior to sequencing by fast atom bombardment (see Results).

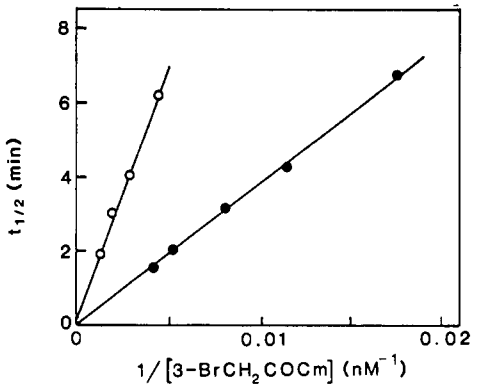


FIGURE 2: Secondary plot of half-time of inactivation ($t_{1/2}$) of CAT as a function of the reciprocal of 3-$BrCH_2COCm$ concentration. Conditions were as described under Experimental Procedures. CAT was incubated with increasing concentrations of 3-$BrCH_2COCm$ in the absence (●) and presence (○) of 30 μM chloramphenicol. Each point represents a 5-min time course, during which time aliquots were removed periodically and assayed for residual CAT activity.

Purification of Type III CAT. The enzyme was purified by the method of Zaidenzaig & Shaw (1976) as described by Packman & Shaw (1981a). The purity and specific activity determinations were as described by Kleanthous & Shaw (1984).

Results

Kinetics of Inactivation of CAT by 3-(Bromoacetyl)-chloramphenicol. The inhibition of CAT by 3-$BrCH_2COCm$ followed pseudo-first-order kinetics. A plot of half-time of inactivation ($t_{1/2}$) vs. the reciprocal inhibitor concentration, according to eq 1 (Meloche, 1967), gave a straight line with

$$t_{1/2} = 1/[3\text{-}BrCH_2COCm]t_{min}K_{inact} + t_{min} \quad (1)$$

intercept close to zero (Figure 2). Inactivation parameters can be deduced in cases where there is a positive intercept on the *y* axis, indicative of rate saturation. K_{inact} represents the inhibitor concentration giving the half-maximal rate of inactivation, and t_{min} is the finite inactivation half-time. For 3-(bromoacetyl)chloramphenicol, the inactivation rate is so rapid that k_{inact} and t_{min} cannot be reliably estimated in this way.

The affinity of the inhibitor for the chloramphenicol binding site/active site was demonstrated by competitive experiments wherein the inactivation was carried out in the presence of chloramphenicol, at a concentration 2–3-fold greater than K_m. Chloramphenicol increases the slope (K_{inact}) while leaving the intercept essentially unchanged (see Figure 2), consistent with chloramphenicol and 3-$BrCH_2COCm$ competing for the same site. This approach was first used by Kitz & Wilson (1962), who showed that the slope of an analogous plot for inactivation of acetylcholinesterase by methanesulfonic acid esters increased in the presence of competitive inhibitors.

Inhibition of Both Acetylation and Hydrolytic Activities of CAT by 3-(Bromoacetyl)chloramphenicol. Incubation of CAT with an excess of 3-$BrCH_2COCm$ abolished all acetylation activity (<1% remaining activity). Thioesterase activity, observed at very high concentrations of enzyme, was lost in parallel with the loss of acetylation activity, supporting the conclusion from kinetic studies that the acetylation of chloramphenicol and hydrolysis of acetyl-CoA are both functions of CAT that occur at the same site on the enzyme surface (Kleanthous & Shaw, 1984).

Stoichiometry of Inactivation. The incorporation of radiolabel into CAT after incubation with increasing concentrations of 3-$BrCH_2$*COCm followed a linear relationship with

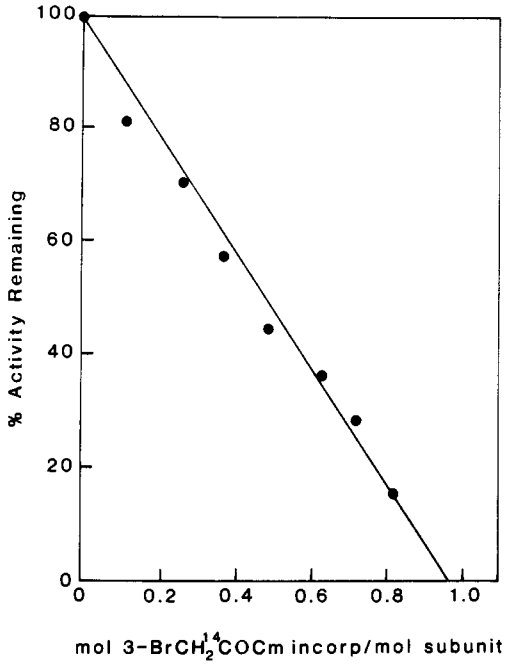


FIGURE 3: Incorporation of 3-$BrCH_2$*COCm as a function of residual CAT acetylation activity. Details are given under Experimental Procedures. Following the incubation of enzyme with a range of concentrations of 3-$BrCH_2$*COCm for 2 min, aliquots were withdrawn for the determination of residual CAT activity and protein-bound radioactivity.

the concomitant loss of enzymic activity as shown in Figure 3. Extrapolation to total inactivation indicates that 0.96 mol of inhibitor was bound per mole of CAT monomer. Experiments aimed at the complete inactivation of CAT, using an excess of either labeled reagent followed by extensive dialysis (24 h), also gave a stoichiometry of 1:1, furthermore, there was no restoration of enzymatic activity, consistent with covalent modification.

Identification and Quantitation of Modified Amino Acids after Inactivation of CAT with 3-(Bromoacetyl)chloramphenicol. Although the inactivation and radioactive labeling experiments gave results compatible with covalent modification of CAT with a 1:1 stoichiometry, it was necessary to show that a unique amino acid residue had been modified. Accordingly, CAT was treated with an excess of 3-$BrCH_2$*COCm and acid hydrolyzed, and the hydrolysate was electrophoresed on a thin-layer silica plate at pH 6.5 (Figure 4). Acid hydrolysis converts α-(halo ester)-modified amino acids to their [^{14}C]carboxymethylated derivatives, which are easily identified and quantitated after electrophoresis (Adamson et al., 1984). Of the most likely nucleophiles within the active site only histidine was modified with no detectable *S*-(carboxymethyl)cysteine present. All of the label (≥90%) was incorporated in the form of N^3-(carboxymethyl)histidine. There was no incorporation in N^1-(carboxymethyl)histidine, nor were there any other radioactive species observed.

Isolation and Characterization of Labeled Peptides. Of the two radiolabeled forms of 3-$BrCH_2$COCm used in this study, only 3-$BrCH_2$*COCm was suitable for the characterization of the modified amino acid in CAT. Although the enzyme–chloramphenicol ester linkage was stable to prolonged dialysis at 4 °C (see stoichiometry experiments), partial loss of [^{14}C]chloramphenicol (when the enzyme was labeled with 3-$BrCH_2$CO*Cm) was observed during the preparation of inactivated CAT for proteolytic digestion (data not shown), notably during the reduction step using DTT in the presence of 6 M guanidine hydrochloride (Zaidenzaig & Shaw, 1978).

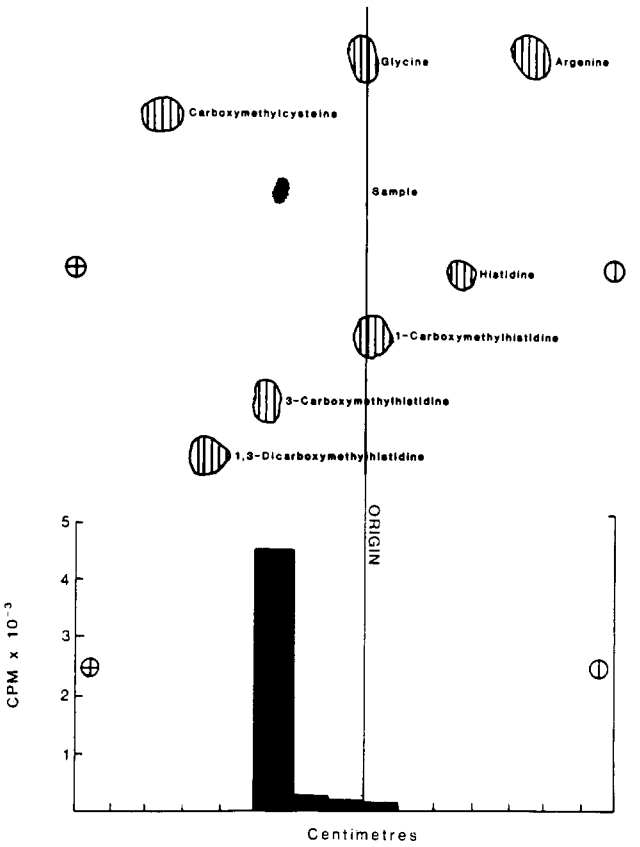


FIGURE 4: High-voltage electrophoresis of acid hydrolysate of CAT inhibited with 3-$BrCH_2$*COCm on a silica plate at pH 6.5; see Experimental Procedures. Two equivalent (5-nmol) samples were subjected to electrophoresis; one track was cut into strips and the incorporated radioactivity determined by scintillation counting; the remainder of the plate was autoradiographed.

Because of the partial and variable hydrolysis and to facilitate the interpretation of peptide maps, the ester was completely hydrolyzed (overnight heating at 40 °C), and the released chloramphenicol was removed by dialysis, leaving [^{14}C]-carboxymethylated protein from which there was no detectable loss of label. The modified and unmodified samples of protein were digested with trypsin and the peptides from each separated by HPLC (Figure 5) on a reverse-phase column. The modified enzyme yielded three altered peptides when compared with the elution profile from the unmodified enzyme (labeled in Figure 5 as TP1, TP2, and TP3). The three peptides also corresponded to the sites of incorporation of radiolabel, the percentage contribution of each peak to the total radioactivity recovered being 13%, 32%, and 55%, respectively. Peaks were collected manually, after large-scale injection (>40 nmol), dried by lyophilization, and desalted. Although sample injections on a preparative scale resulted in some loss of resolution, the fractions collected were of sufficient purity to be sequenced directly by a strategy of proteolytic digestion and manual Edman degradation coupled with fast atom bombardment mass spectrometry (FAB mapping; Morris et al., 1983). The details of the methods used will be published elsewhere (M, Panico, C. Kleanthous, and H. R. Morris, unpublished results). Each fraction was digested with chymotrypsin, resulting in the loss of the predominant mass ions for peptides TP2 and TP3 but the appearance of a signal identical with that of TP1 (1876 daltons). All three fractions appeared, therefore, to be related to one another and almost certainly represent partial cleavage products from the original tryptic digestion. TP1 (and TP2/TP3 after chymotrypsin treatment) was subjected to six rounds of Edman cleavage,

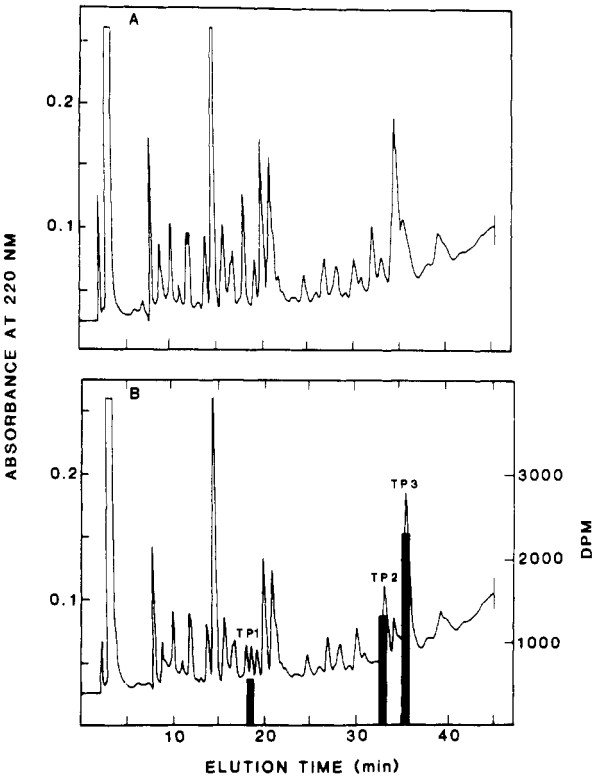


FIGURE 5: Reverse-phase high-pressure liquid chromatographs of tryptic digests of (A) unmodified CAT and (B) CAT modified with 3-$BrCH_2$*COCm. The preparation of samples and the conditions of elution for HPLC mapping are described under Experimental Procedures. In both cases, 5–6 nmol of material was injected. Peaks were collected manually, and radioactivity was located by scintillation counting.

which identified the N-terminal residues as Ser-Val-Gln-Val-His-His and located the site of carboxymethylation to the second histidine. Carboxypeptidase B treatment of TP1 (as well as TP2 and TP3) yielded arginine as the C-terminal residue. The complete amino acid sequence of TP1 was deduced by relating its molecular weight (from fast atom bombardment mass spectrometry), amino-terminal sequence, and C-terminal arginyl residue to the primary structure of the type III variant of CAT (Shaw, 1983; I, Murray, A. Hawkins, and W. V. Shaw, unpublished results). The proposed sequence of TP1 (with numbering from the complete structure) is thus

Ser-Val[185]-Gln-Val-
His-His-Ala[190]-Val-Cys-Asp-Gly-Phe[195]-His-Val-Ala-Arg

The presence of N^3-(carboxymethy)histidine at the sixth position (from inactivation by 3-$BrCH_2$COCm) and one *S*-(carboxymethyl)cysteine (via alkylation of the denatured protein prior to tryptic digestion) yields a theoretical molecular weight for the above peptide of 1876, exactly equal to the observed molecular weight of TP1. The identity of the peptide was confirmed by the observed molecular mass (1413 daltons) of the major chymotryptic fragment of TP1, the N-terminal dodecapeptide ending at Phe-195.

Discussion

Affinity labeling of CAT by 3-$BrCH_2$COCm was clearly demonstrated by the protection against inactivation afforded by chloramphenicol. In conjunction with the very rapid inactivation rate, the data provide good evidence for highly efficient active site directed inhibition in which specific binding at the chloramphenicol site increases the local concentration of inhibitor at the catalytic center. Subsequent to analogue binding, the favorable juxtaposition of the reactive imidazole group relative to the bromoacetyl functionality promotes extremely rapid inactivation.

Hirtherto, there has been little direct evidence to support the belief that histidine has a catalytic function, as opposed to involvement in binding the substrates. The close proximity of the bromoalkyl group of 3-$BrCH_2$COCm to the normal site of reaction (the ester carbonyl) provides the clearest evidence thus far in favor of a mechanistic role for the uniquely reactive histidine.

The rate of inactivation is extremely rapid. As a result, the highest convenient concentration of inhibitor in these experiments was 0.24 μM, for which the half-time for inactivation is less than 2 min. This concentration is several orders of magnitude below the expected binding constant (13 μM) for the substrate for the reverse reaction, 3-acetylchloramphenicol. Kitz & Wilson (1962) have pointed out that, under such conditions, the observed rate of inactivation must be proportional to the inhibitor concentration, even if a reversible complex is formed prior to inactivation. The very rapid rate of inactivation, therefore, prevents detection of rate saturation. The rate of inactivation of triosephosphate isomerase by haloacetol phosphates (Hartman, 1971) was also shown to be too fast for the detection of rate saturation. The apparent bimolecular rate constant was 2600 M^{-1} s^{-1} at 2 °C and pH 6.5 for the inhibition by bromoacetol phosphate. In comparison, the apparent bimolecular rate constant for the inhibition of CAT by 3-$BrCH_2$COCm at 25 °C and pH 7.5 was 30×10^3 M^{-1} s^{-1}.

The magnitude of the bimolecular rate constant can be placed in perspective when compared with that measured for a nonspecific reagent such as iodoacetamide. Iodoacetamide possesses a similar electrophilic group to 3-$BrCH_2$COCm but lacks the structural features necessary for specific association at the chloramphenicol binding site such as the propanediol side chain or the nitrophenyl group. Apparent bimolecular rate constants for iodoacetamide inactivation, calculated from the data of Fitton & Shaw (1979) and Corney (1983), respectively, were 0.06 M^{-1} s^{-1} for the staphylococcal CAT variants and 0.015 M^{-1} s^{-1} for CAT type III. The apparent bimolecular rate constants for the nonspecific reagent are approximately 6 orders of magnitude lower than that observed with 3-$BrCH_2$COCm. This enhanced reactivity, or facilitation (Plapp, 1982), might reasonably be expected if the analogue was fulfilling the protein–substrate contacts anticipated for chloramphenicol when bound to CAT and so orienting the electrophilic bromoacetyl group toward a reactive nucleophile within the catalytic center. The primary function of CAT is to confer bacterial resistance to the antimicrobial activity of chloramphenicol by catalyzing the acetylation of the antibiotic, utilizing acetyl-CoA as the acyl donor. However, at elevated concentrations of enzyme (micromolar and above), the hydrolysis of a variety of acyl thio esters of CoA has been observed [for a discussion of the kinetics of this hydrolytic activity, see Kleanthous & Shaw, (1984)]. This suggests that CAT is capable of catalyzing the transfer of an acyl group not only to chloramphenicol but also to water, albeit less efficiently. Stoichiometric alkylation with 3-$BrCH_2$COCm abolished all acetylation and hydrolytic activity associated with CAT, consistent with the view that both functions take place at the same site.

Although completely stable in organic solvents, 3-$BrCH_2$COCm is subject to hydrolysis in aqueous solution. The spontaneous hydrolysis of the compound in buffer (pH 7.5 and 25 °C), to chloramphenicol and bromoacetate, was monitored

by HPLC (Kleanthous & Shaw, 1984), yielding a hydrolytic rate constant of 0.01 min^{-1} ($t_{1/2}$ = 77 min). Typical incubation times used in this study (no greater than 5 min) resulted in the loss of less than 5% of the inhibitor (data not shown). Since substrate levels of CAT are known to hydrolyze thio esters of CoA, the possibility existed that the α-(halo ester) of 3-$BrCH_2COCm$ might also be a substrate for hydrolysis. This was tested by the inactivation of CAT with an excess of reagent, extraction of the unbound material with ethyl acetate, and its analysis by TLC (see Experimental Procedures). There was no increase in the level of free chloramphenicol after incubation of the reagent with CAT relative to that for buffer alone, thus confirming the absence of enzyme-catalyzed hydrolysis of 3-$BrCH_2COCm$ (data not shown).

Since 3-acetylchloramphenicol is known to isomerize to 1-acetylchloramphenicol at a rate of approximately 0.04 min^{-1} with an equilibrium constant of 0.38 at pH 7.5 and 25 °C (Kleanthous & Shaw, 1984), the possibility existed that CAT might be inactivated by 1-$BrCH_2COCm$ following the analogous rearrangement of 3-$BrCH_2COCm$. Although the NMR spectrum of recrystallized 3-$BrCH_2COCm$ showed no evidence of the 1-acetyl isomer, when diluted into buffer and analyzed by HPLC, isomerization to 1-$BrCH_2COCm$ (~15%) was detected (data not shown). The significance of the 1-acetyl isomer as an alkylating agent was tested by synthesizing the 1-(bromoacetyl)-3-fluorochloramphenicol analogue, on the basis of the competitive inhibitor of CAT 3-fluorochloramphenicol (K_i = 78 μM), wherein migration of the bromoacetyl moiety to the 3-position is blocked by the fluorine atom. No inhibition was seen with this compound, even at millimolar concentrations, a result consistent with alkylation occurring only with the bromoacetyl group at the 3-position.

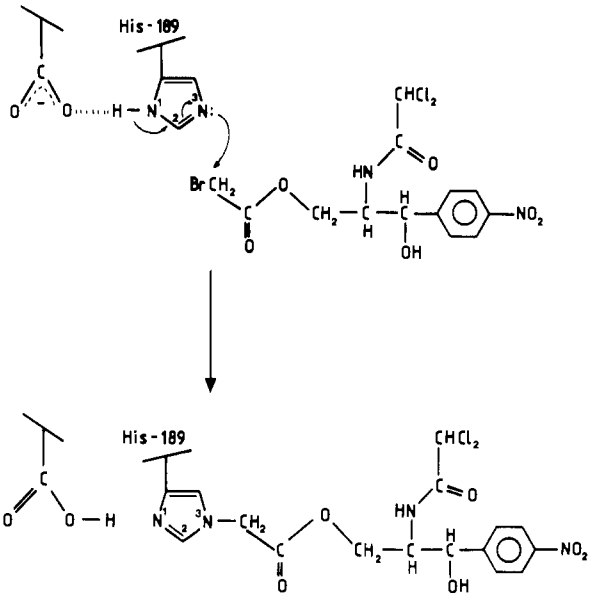
3-(Bromoacetyl)chloramphenicol uniquely alkylates His-189 in the type III variant of CAT (corresponding to His-193 when aligned with the primary structure of the type I enzyme) with the consequent loss of acetylation and hydrolytic activity. Among the CAT variants sequenced to date [for a review, see Shaw (1983)], all possess a highly conserved region:

Gln-Val-His-His*-Ala-Val-Cys-Asp-Gly

which encompasses the highly reactive histidine residue (*) modified by 3-$BrCH_2COCm$. The assignment of His-189 as the site of alkylation by the affinity-labeling reagent provides good evidence that this is indeed the active site, particularly in conjunction with the high degree of conservation in the surrounding amino acid sequence. Indeed, the assignment of this segment of the primary structure to the active site has prompted the use of an oligodeoxyribonucleotide (20-mer) as a specific probe for CAT genes in other organisms (Beschle et al., 1984).

Histidine modification in proteins frequently shows preference for one of the two possible tautomeric forms (Vogel & Bridger, 1983). Methylation of the catalytically important histidine residues in both chymotrypsin and phospholipase A_2 by methyl *p*-nitrobenzenesulfonate (Nakagawa & Bender, 1970; Verkij et al., 1980) resulted in specific modifications at the N^3 and N^1 positions, respectively. In both cases, crystallography identified a hydrogen bond to an aspartic residue as the necessary stabilization for each tautomer (Blow et al., 1969; Verkij et al., 1980). Methylation of the histidine in D-amino acid oxidase, however, resulted in both N^3- and N^1-methylhistidine, a result that was postulated as evidence for the lack of tautomer stabilization (Swenson et al., 1984). In the present study, thin-layer electrophoresis unambiguously identifies N^3-(carboxymethyl)histidine as the only modified form of histidine after inactivation by 3-$BrCH_2COCm$, a result

Scheme II



compatible with the existence of a histidine–carboxylate hydrogen-bonded couple within the active site of CAT. Similarly, Adamson et al. (1984) have postulated a histidine–aspartate couple within the E3 portion of the pyruvate dehydrogenase multienzyme complex on the basis of the identification of N^3-(carboxymethyl)histidine, after modification, by high-voltage paper ionograms.

In principle, His-189 could function as a nucleophile or a general base in the acetyl-transfer reaction. The kinetic data strongly favor a mechanism involving a ternary complex (sequential mechanism) rather than a true ping-pong mechanism involving an acetyl-enzyme intermediate (Kleanthous & Shaw, 1984). Consequently, the role of His-189 in CAT is most likely that of a general base. The evidence, to date, suggests that the nitrogen, in position 3 of the imidazole ring, abstracts a proton from the primary hydroxyl of chloramphenicol or from water (in the absence of ligand), promoting nucleophilic attack of the thio ester of acetyl-CoA. Furthermore, since 3-$BrCH_2COCm$ is two carbon units longer than chloramphenicol, it must be assumed that the bromoacetyl side chain is free to adopt an appropriate conformation that allows reaction with the catalytic histidine, as depicted in Scheme II.

The specificity and extremely rapid reaction of 3-$BrCH_2COCm$ with the active site of CAT will facilitate the use of this reagent for active site titration. Furthermore, since CAT activity constitutes the principle form of bacterial resistance to chloramphenicol, 3-(bromoacetyl)chloramphenicol represents the prototype for the design of drugs capable of overcoming enzymatic resistance, analogous to the role of β-lactamase inhibitors in overcoming resistance to β-lactam antibiotics.

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Turkey Ovomucoid Third Domain Inhibits Eight Different Serine Proteinases of Varied Specificity on the Same ...Leu[18]-Glu[19]... Reactive Site†

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ABSTRACT: We show that eight different serine proteinases—bovine chymotrypsins A and B, porcine pancreatic elastase I, proteinase K, *Streptomyces griseus* proteinases A and B, and subtilisins BPN′ and Carlsberg—interact with turkey ovomucoid third domain at the same Leu[18]–Glu[19] peptide bond, the reactive site of the inhibitor. Turkey ovomucoid third domain was converted to modified (the reactive site peptide bond hydrolyzed) form as documented by sequencing. Complexes of all eight enzymes both with virgin and with modified inhibitor were prepared. All 16 complexes were subjected to kinetically controlled dissociation, and all 16 produced predominantly virgin (>90%) inhibitor, thus proving our point. During this investigation, we found that both α-chymotrypsin and especially *S. griseus* proteinase B convert virgin to modified turkey ovomucoid third domain, even in the pH range 1–2, a much lower pH than we expected. We have also measured rate constants k_{on} and k_{on}^* for the association of virgin and modified turkey ovomucoid third domain with several serine proteinases. The k_{on}/k_{on}^* ratio is 4.8×10^6 for chymotrypsin, but it is only 1.5 for subtilisin Carlsberg. A number of generalizations concerning reactive sites of protein proteinase inhibitor are proposed and discussed.

The mechanism of interaction of serine proteinases with protein proteinase inhibitors is now clearly established (Laskowski & Kato, 1980). Its simplest form, first formulated by Finkenstadt & Laskowski (1967), can be given by the equation

$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} C \underset{k_{on}^*}{\overset{k_{off}^*}{\rightleftharpoons}} E + I^* \qquad (1)$$

where E is the proteinase, I and I* are the virgin (reactive site peptide bond intact) and modified (reactive site peptide bond hydrolyzed) inhibitors, respectively, C is the stable complex, k_{on} and k_{on}^* are the second-order rate constants for association of the enzyme with the virgin and modified inhibitor, respectively, and k_{off} and k_{off}^* are the first-order dissociation rate constants. According to the above mechanism, the stable enzyme–inhibitor complex, C, is the same substance whether it is made from virgin inhibitor, I, or from modified inhibitor, I*. A useful test for this has been designed. It is called "kinetically controlled dissociation". The complexes are made at neutral pH by mixing equimolar quantities of enzyme and inhibitor and allowing sufficient time for the association. The pH is then suddenly lowered to a very low value. At neutral pH, the equilibrium largely favors complex over dissociated products. At very low pH, the complex is not favored; it must dissociate. At low pH, the values of k_{on} and k_{on}^* are negligible

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