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A Protease Activity Associated with Acetylcholinesterase Releases the Membrane-Bound Form of the Amyloid Protein Precursor of Alzheimer's Disease†

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ABSTRACT: Amyloid deposits in the brains of patients with Alzheimer's disease (AD) contain a protein (βA4) which is abnormally cleaved from a larger transmembrane precursor protein (APP). APP is believed to be normally released from membranes by the action of a protease referred to as APP secretase. Amyloid deposits have also been shown to contain the enzyme acetylcholinesterase (AChE). In this study, a protease activity associated with AChE was found to possess APP secretase activity, stimulating the relase of a soluble 100K form of APP from HeLa cells transfected with an APP cDNA. The AChE-associated protease was strongly and specifically inhibited by soluble APP (10 nM) isolated from human brain. The AChE-associated protease cleaved a synthetic βA4 peptide at the predicted cleavage site. As AChE is decreased in AD, a deficiency of its associated protease might explain why APP is abnormally processed in AD.

Alzheimer's disease (AD) is characterized by deposition of amyloid in the intracellular and extracellular compartments of the cerebral cortex. The extracellular amyloid consists of a protein (βA4) of 4000 relative molecular mass (M_r = 4K). βA4 comprises part of the membrane-spanning and extracellular domains of a much larger precursor protein (APP) of 110-130K, which has features of an integral transmembrane cell surface receptor (Kang et al., 1987). At least three APP isoforms, produced by alternative mRNA splicing, contain an extra 56-residue domain similar to Kunitz type protease inhibitors (KPI) (Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988).

Proteolysis of APP results in the secretion of a 100-110K ectodomain, the KPI-containing forms of which are identical to protease nexin II (Oltersdorf et al., 1989; Van Nostrand et al., 1989), a protease inhibitor that may be involved in neurotrophic mechanisms. Recent studies suggest that the normal cleavage of APP occurs at or near a lysine residue within the βA4 sequence (Esch et al., 1990). Cleavage of this site would prevent formation of the amyloidogenic βA4 fragment. The protease which cleaves at this site ("APP secretase") has not been identified, but a deficiency in this enzyme might lead to abnormal processing of APP and production of amyloidogenic βA4.

The cholinergic system is vulnerable in AD, resulting in a reduction in the cholinergic enzymes acetylcholinesterase (AChE) (Davies & Maloney, 1976; Davies, 1979; Fishman et al., 1986; Hammond & Brimijoin, 1988) and choline acetyltransferase (Bowen et al., 1976; Davies & Maloney, 1976). Despite the substantial depletion of AChE in the AD brain, the enzyme accumulates within both amyloid plaques (Friede, 1965; Struble et al., 1982) and tangles (Mesulam & Moran, 1987; Carson et al., 1991). An AChE-associated protease (AChE-AP) with both trypsin-like and carboxypeptidase activities is recovered with AChE purified by affinity chromatography from tissues rich in AChE such as fetal bovine serum or eel electroplax organ (Small et al., 1987; Small, 1988). The trypsin-like activity of AChE-AP is associated with a 25K protein which may bind to AChE or may be a fragment of AChE produced through proteolysis of an AChE catalytic subunit (Small & Simpson, 1988; Small, 1990).

The substrate for the AChE-AP has not been identified. In this paper, we provide evidence that the AChE-AP may possess an "APP secretase" function, cleaving APP from the cell membrane.

Materials and Methods

Materials. Purified human brain APP was prepared from a soluble extract of post-mortem human brain grey matter by procedures involving heparin-Sepharose, ion-exchange, and dye-ligand chromatography (Moir et al., in preparation). Enzyme inhibitors (aprotinin, leupeptin, soybean trypsin inhibitor, and BW284C51) and crude eel AChE (type V-S) were

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all from Sigma Chemical Co. (St. Louse, MO). α_1-Antichymotrypsin from human plasma was from Calbiochem (San Diego, CA).

Purification of AChE-AP. AChE-AP activity was purified from eel electroplax organ, from fetal bovine serum, or from human brain by chromatography using an affinity resin specific for AChE. For purification of serum or eel AChE-AP, fetal bovine serum (1–5 L) or a commercial preparation of eel AChE (1.0 mg in 1 mL) was applied to a 2.5 × 2.0 cm column of edrophonium–Sepharose prepared as previously described (Small et al., 1987). The column was washed with 100 mL of 50 mM Tris-HCl buffer, pH 7.4 (Tris buffer), containing 0.15 M sodium chloride. AChE-AP was eluted with 30 mL of 30 mM edrophonium chloride in Tris buffer. The affinity eluates were concentrated by using CF25 filter cones (Amicon) and then further purified to apparent homogeneity by size-exclusion HPLC on Bio-Sil TSK400 SW (Small et al., 1987). This purification scheme resulted in a 110000-fold and 20-fold purification of AChE activity from fetal bovine serum and from the commercial preparation, respectively.

Human brain protease was purified approximately 400000-fold from a soluble extract of post-mortem human brain gray matter. Brain tissue (750 g) was homogenized on ice in 500 mL of Tris buffer containing 0.15 M sodium chloride using an Ultra-Turrax T-25 and then centrifuged at 11000 rpm, at 4 °C for 30 min in a JA rotor using a Beckman J2-21M/E centrifuge. The supernatant fraction was collected and centrifuged at 30000 rpm, at 4 °C for 60 min in a 50.2Ti rotor using a Beckman L8-80M ultracentrifuge. The supernatant fraction was collected and applied to the edrophonium–Sepharose affinity column and further purified as described for serum and eel AChE-AP.

Culture of HeLa Cells and Transfection with APP_{695} cDNA. HeLa cells were transiently transfected by calcium phosphate coprecipitation with a plasmid (pAD-695) encoding the 695 amino acid form of APP (Weidemann et al., 1989). The following day, cells were subcultured into 24-well plastic culture dishes at a density of 10^5 cells per well in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. After 1 day, the medium was removed and the cells in each well washed with 2 × 1 mL serum-free medium. The cells were then incubated with 200 μL/well serum-free medium containing proteases or the protease inhibitor aprotinin. After 1-h incubation, the conditioned medium was removed and concentrated to 25 μL and each sample assayed for APP immunoreactivity by Western blotting.

A crude membrane fraction of HeLa cells was obtained by scraping the cells from one well into 1.0 mL of 50 mM Tris-HCl, pH 7.4. The cells were lysed by sonication with five bursts using a Branson B15 cell disruptor at 50% intermittency. A membrane fraction was obtained by centrifugation at 13,000 rpm for 20 min at 4 °C using a Heraeus Sepatech Biofuge.

APP Assay. The amount of APP secreted from HeLa cells was determined quantitatively using a slot–blot assay. Conditioned media (200 μL) from HeLa cell incubations were centrifuged (500 g, 15 min) to remove any cell debris and then blotted directly onto nitrocellulose sheets using a slot–blot apparatus (Bio-Rad, Richmond, CA). Each slot was washed twice with 500 μL of 50 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, and then nitrocellulose sheets were stained for APP immunoreactivity using a mouse monoclonal antibody (22C11) (Weidemann et al., 1989), using bromochloroindolyl phosphate and nitro blue tetrazolium as substrate for an alkaline phosphatase conjugated secondary antibody. The density of staining was determined using a scanning laser densitometer with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Purified human brain APP (0–1 μg) was used as standard in the assay.

Digestion of a Synthetic βA4 Peptide by AChE-AP. The digestion of a synthetic fragment of APP ($\beta A4_{10-23}$) was monitored by reversed-phase high-performance liquid chromatography (RP-HPLC) on a Novapak C18 column (0.4 × 150 mm) (Waters) using a Bio-Rad Model 700 HPLC. The synthetic C-terminally amidated peptide (YEVHHQKLVFFAED-NH_2) was synthesized by the Fmoc procedure (Atherton et al., 1981). Synthetic peptide (50 μg) and AChE-AP fractions were incubated in a total volume of 1.0 mL of Tris buffer. The digestion was stopped by adding 0.1 mL of glacial acetic acid and the digestion mixture applied to the reversed-phase column, which was preequilibrated with 0.1% (v/v) trifluoroacetic acid in water. The column was eluted with a linear gradient of 0–60% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid/water over 60 min. The effluent was monitored at 215 nm. Peptides were identified by amino acid analysis and by comparison with the elution times of the two fragments generated by tryptic digestion of the synthetic peptide.

Gel Electrophoresis and Western Blotting. Fractions were analyzed for APP immunoreactivity by Western blotting onto nitrocellulose following 10% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Nitrocellulose sheets were stained by a mouse monoclonal antibody (22C11) with an alkaline phosphatase conjugated secondary antibody using naphthol AS-MX and Fast Red as substrate (Bush et al., 1990).

Enzyme Assays. To study the effects of inhibitors on the protease activity, a model peptide substrate (Leu-Trp-Met-Arg-Phe-Ala) was incubated with enzyme fractions and the amount of tryptic product (Leu-Trp-Met-Arg) assessed by RP-HPLC as previously described (Small et al., 1987). Units of protease activity were micrograms of model peptide cleaved per hour at 37 °C. AChE activity was assayed by the method of Ellman et al. (1961). Units of esterase activity were micromoles of acetylthiocholine hydrolyzed per minute at 30 °C.

Protein Assay. Protein was assayed using the method of Bradford (1974) with bovine serum albumin as standard.

Results

Effect of AChE-AP on Secretion of APP from HeLa Cells. We examined the ability of the AChE-AP to cleave APP from the membranes of HeLa cells transfected with a cDNA encoding the 695 amino acid form of APP. HeLa cells were incubated with preparations of AChE-AP (5 μg/mL). After incubation, the release of APP was assessed by Western blotting using a monoclonal antibody specific for the APP ectodomain (Figure 1).

HeLa cell membranes contained a single major species of APP of 110K (Figure 1, lane 1). As previously reported (Weidemann et al., 1989), the cells release a 100K form of APP into the conditioned medium (lane 4) presumably as a result of proteolytic cleavage of the 110K transmembrane form (Palmert et al., 1989; Weidemann et al., 1989; Esch et al., 1990; Sisodia et al., 1990). Incubation of the cells with AChE-AP stimulated the release of this 100K form (lanes 2 and 7). The stimulation by the AChE-AP fraction was due to its proteolytic action, as preincubation of the AChE-AP with aprotinin abolished the effect (lane 3). Neither bovine pancreatic chymotrypsin (0.1 μg/mL) nor bovine pancreatic trypsin (0.1 μg/mL) stimulated the release of a 100K form of APP (lanes 5 and 6), as APP was not detected in the conditioned medium. The failure to detect any endogenously

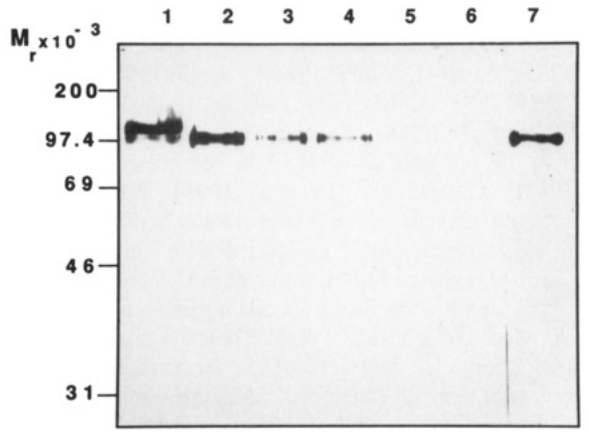

FIGURE 1: Western blot analysis of the release of APP immunoreactivity from transiently APP_{695}-transfected HeLa cells by an AChE-associated protease (AChE-AP). Cells were transfected with plasmid (pAD-695) and then incubated in the presence of various proteases. Blots were stained with a monoclonal antibody (22C11) which recognizes the APP ectodomain. The figure shows the release of a 100K polypeptide after incubation with AChE-AP. Lane 1, HeLa membrane fraction; lanes 2–7, conditioned medium with no addition (lane 4) or in the presence of serum AChE-AP (5 μg/mL) (lane 2), serum AChE-AP + aprotinin (80 μg/mL) (lane 3), eel AChE-AP (5 μg/mL) (lane 7), bovine pancreatic trypsin (0.1 μg/mL) (lane 5), and bovine pancreatic chymotrypsin (0.1 μg/mL) (lane 6). The amount of trypsin used yielded the identical amount of tryptic activity as AChE-AP as assessed using $\beta A4_{10-23}$ as substrate (Figure 3). The positions of molecular weight markers (myosin, 200K; phosphorylase, 97.4K; bovine serum albumin, 69K; ovalbumin, 46K; carbonic anhydrase, 31K) are shown.

secreted APP in these incubations may have resulted because both proteases cleave APP within the epitope recognized by the monoclonal antibody. APP secretion was not stimulated when HeLa cells were incubated with lower concentrations of trypsin or chymotrypsin (data not shown).

To more accurately quantitate the amount of APP released from HeLa cells by the AChE-AP, the amount of APP released from transfected and untransfected cells was measured by immunoassay on nitrocellulose membranes using a slot–blot assay on nitrocellulose membranes (Figure 2). The amount of staining was determined by laser scanning densitometry, and the values obtained were compared with those obtained using purified human brain APP as the standard. APP_{695}-transfected cells released a 6-fold greater amount of immunoreactive APP than untransfected cells. In the presence of AChE-AP (10 μg/mL), the release of APP immunoreactivity was stimulated 3-fold and 7-fold for the untransfected and transfected cells, respectively.

Cleavage of βA4 Peptide by AChE-AP. Release of APP from cells may follow proteolysis at a putative cleavage site adjacent to lysine-16 of the βA4 sequence. To examine whether the AChE-AP cleaved at this site, a peptide ($\beta A4_{10-23}$) containing the sequence surrounding the proposed cleavage site was incubated with the AChE-AP purified from eel and fetal bovine serum and the digestion monitored by RP-HPLC (Figure 3). After 1 h of incubation, $\beta A4_{10-23}$ was digested by the AChE-AP to yield two peptides, which were identified by amino acid analysis as the N- and C-terminal fragments of $\beta A4_{10-23}$ produced by cleavage on the C-terminal side of the lysine residue. The identity of the two peptides was confirmed by comparing their elution times with those obtained by digesting $\beta A4_{10-23}$ with trypsin, which cleaves on the C-terminal side of lysine residues.

Presence of AChE-AP Activity in Human Brain. Human brain contains less than 1% of the soluble AChE activity of that present in fetal bovine serum (Gennari & Brodbeck,

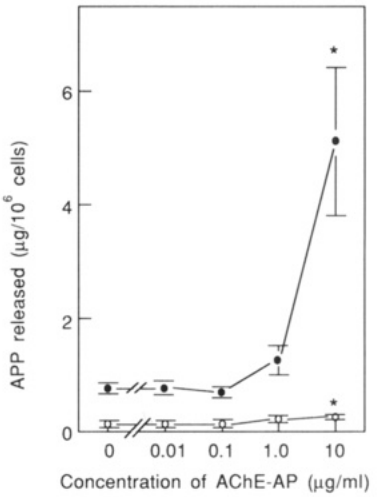

FIGURE 2: Secretion of APP immunoreactivity from untransfected (○) and APP_{695}-transfected (●) HeLa cells. Cells were cultured at a density of 10^5 cells/well in 24-well dishes and then incubated with eel AChE-AP. The amount of APP secreted was measured using a slot–blot immunoassay. Each point represents the mean value obtained from four incubations. Error bars show the SEM. Asterisks (*) show values that are significantly different ($P < 0.05$) from the corresponding control value (0 μg/mL AChE-AP) as assessed using a two-tailed Student's t test.

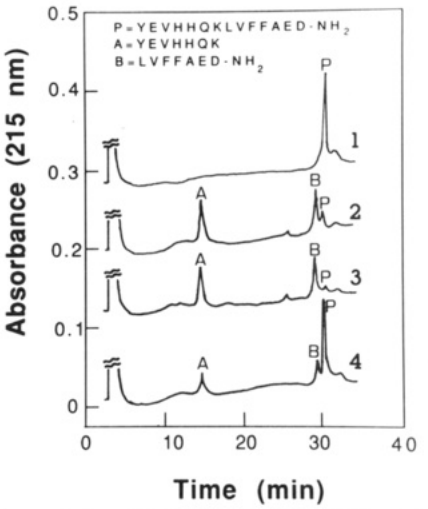

FIGURE 3: Reversed-phase HPLC of an AChE-AP digest of a synthetic peptide fragment of APP ($\beta A4_{10-23}$) on a Novapak C18 column (0.4 × 150 mm) (Waters) using a Bio-Rad Model 700 HPLC. The synthetic peptide (P) was incubated without protease (incubation 1) or in the presence of serum AChE-AP (incubation 2) or eel AChE-AP (incubation 3) for 1 h. In incubation 4, peptide was incubated with 400 000-fold purified human brain AChE for 24 h. Control experiments confirmed that the peptide was completely stable over 24 h in the absence of any added proteases. The identities of the two peptide products (A and B) were confirmed by amino acid analysis and by comparison with the elution times of the same two peptides generated by digestion with trypsin.

1985). Nonetheless, we examined the possibility that human brain AChE might also possess an associated protease activity. AChE was purified 400 000-fold from a soluble extract of human brain gray matter by affinity chromatography and then incubated with the $\beta A4_{10-23}$ peptide. A protease activity similar to that associated with the eel and bovine serum AChEs was identified in the human brain fraction, as the $\beta A4_{10-23}$ peptide was slowly cleaved to yield the same two peptide products as those obtained in the incubations with the serum and eel preparations (Figure 3).

Effect of Serine Protease Inhibitors. Proteases that process APP may be inhibited by the KPI domain within the protein.

Table I: Effect of Serine Protease and Esterase Inhibitors on Enzyme Activities Associated with Acetylcholinesterase[a]

	eel AChE		serum AChE	
treatment	esterase activity	protease activity	esterase activity	protease activity
control	10800 ± 1400	740 ± 80	3000 ± 230	5650 ± 1800
APP	11700 ± 1000	160 ± 10*	3030 ± 560	260 ± 40*
aprotinin	11900 ± 300	90 ± 30*	2930 ± 320	1230 ± 700*
SBTI	12300 ± 700	700 ± 60	2720 ± 170	3920 ± 1700
leupeptin	10300 ± 1400	900 ± 100	2490 ± 460	5780 ± 150
α_1-ACT	9400 ± 900	810 ± 30	1430 ± 320*	5200 ± 350
BW284C51	1200 ± 200*	755 ± 70	150 ± 30*	5200 ± 1900

[a] AChE (2.5 μg of protein/mL) purified from fetal bovine serum or eel electric organ was preincubated with human brain amyloid precursor protein (APP), bovine lung aprotinin, soybean trypsin inhibitor (SBTI), leupeptin, α_1-antichymotrypsin (α_1-ACT), or the AChE inhibitor BW284C51 (10 μM) for 1 h at 37 °C in 50 mM Tris-HCl buffer, pH 7.4. Aliquots (50 μL) from each incubation were assayed for esterase activity (Ellman et al., 1961) or trypsin-like protease activity (Small et al., 1987). The concentration of protease inhibitors was 10 nM. The molecular weight of APP was assumed to be 80000 for purposes of calculation. Values are means ± SD ($n = 4$). Asterisks (*) show values significantly different from controls ($P < 0.05$) using a two-tailed Student's t test. Amyloid protein precursor (APP) was purified from human brain by a combination of heparin–Sepharose, ion-exchange, and dye–ligand chromatography (manuscript in preparation). Units of esterase activity are micromoles of acetylthiocholine hydrolyzed per minute at 30 °C. Units of protease activity are micrograms of model peptide (Leu-Trp-Met-Arg-Phe-Ala) cleaved per hour at 37 °C.

We found that purified human brain APP (10 nM) inhibited the eel and serum AChE-APs approximately 80% and 95%, respectively (Table I). The serine protease inhibitor aprotinin, which shares approximately 50% amino acid sequence similarity to the KPI domain of APP, also inhibited the protease activity, whereas the other serine protease inhibitors, including the broad-spectrum inhibitors leupeptin and soybean trypsin inhibitor, did not inhibit at this concentration. With the exception of α_1-antichymotrypsin which was a weak inhibitor of the esterase activity of serum AChE, the protease inhibitors did not inhibit the esterase activity of AChE using these incubation conditions, although this activity was strongly inhibited by the specific AChE inhibitor BW284C51.

Discussion

This study demonstrates that a protease activity associated with AChE (AChE-AP) can stimulate the release of APP from HeLa cells. This action by the AChE-AP probably results from cleavage of the protein at or close to a site predicted to be the site of normal proteolytic cleavage by APP secretase, the enzyme which liberates APP from membranes by cleaving within the βA4 sequence (Esch et al., 1990). Studies confirmed that the AChE-AP can cleave a model peptide ($\beta A4_{10-23}$) at a position that corresponds to the APP secretase cleavage site in the full-length protein. Our study also found that soluble APP isolated from human brain was a potent inhibitor of the AChE-AP, suggesting that, upon release from the membrane, APP may inhibit the protease activity which releases it.

AChE has been reported to be present in both amyloid plaques and neurofibrillary tangles in the brains of patients with AD (Friede, 1965; Mesulam & Moran, 1987; Carson et al., 1991). Primate studies have shown that AChE may be deposited in plaques during the early stages of amyloid formation (Struble et al., 1982). In mature plaques, AChE is predominantly associated with the amyloid, rather than the neuritic component, while in tangle-rich regions of temporal cortex, AChE is localized to the neurofibrillary tangle (Carson et al., 1991).

The presence of AChE in association with amyloid has yet to be explained. One possibility is that deposition of AChE results from the degeneration of cortical cholinergic neurons. The loss of AChE-containing neurons in the cortex, hippocampus, and basal nucleus commonly occurs in AD (Davies & Maloney, 1976; Davies, 1979; Fishman et al., 1986; Hammond & Brimijoin, 1988). Recently, an anomalous molecular form of AChE has been detected in the cerebrospinal fluid of patients with AD (Navaratnam et al., 1991). While the loss of cholinergic neurons in the AD brain has been well documented, not all amyloid is associated with cholinergic or cholinoceptive regions. For example, depletion of AChE-rich pyramidal neurons in the neocortex and hippocampus has been reported (Mesulam & Geula, 1988). These pyramidal neurons are not cholinergic as they lack choline acetyltransferase, nor is it likely that they receive direct cholinergic input, as they do not respond directly to cholinergic agents (Reece & Schwartzkroin, 1991).

However, AChE may have a more fundamental role in amyloid formation. Loss of cellular AChE and deposition of AChE within amyloid plaques may be due to the degeneration of AChE-containing neurons, some of which may not be cholinergic. The association of protease activity with AChE in vitro suggests that protease activity may also be associated with the enzyme in vivo. In AD, loss of AChE and its associated protease activity may result in localized loss of ability to cleave APP within the βA4 sequence, as a consequence increasing the concentration of a full-length (potentially amyloidogenic) form of APP.

It has been suggested that KPI-containing forms of APP may inhibit the normal proteolytic degradation of APP, resulting in the deposition of cerebral amyloid (Müller-Hill & Beyreuther, 1989; Selkoe, 1990). In this regard, the finding that soluble APP is a potent and specific inhibitor of the AChE-AP may be of relevance. Levels of KPI-containing forms of APP may be increased in AD (Johnson et al., 1990), resulting in inhibition of APP secretase and the subsequent release of full-length APP.

As human brain AChE may also possess an associated protease activity similar to that found in AChEs purified from richer sources of the enzyme (Figure 3), it will be important to isolate and further characterize the human form of AChE-AP and to examine whether it is deficient in either the familial or sporadic forms of AD. Correction of this deficiency may have therapeutic implications.

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Registry No. Acetylcholinesterase, 9000-81-1; proteinase, 9001-92-7.

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Intrinsic Fluorescence of Chloramphenicol Acetyltransferase: Responses to Ligand Binding and Assignment of the Contributions of Tryptophan Residues by Site-Directed Mutagenesis†

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ABSTRACT: Replacement by tyrosine or phenylalanine was used to assign the additive contributions of each of the three tryptophan residues of chloramphenicol acetyltransferase (CAT) to its intrinsic fluorescence on excitation at 295 nm. During the assessment of the fluorescence responses of the wild-type enzyme to the binding of ligands, it was found that the overlapping absorption spectra of chloramphenicol and tryptophan, with an attendant inner filter effect, required the use of a displacement technique involving an alternative substrate (the *p*-cyano analogue of chloramphenicol) without significant absorption at 295 nm. By the use of two-Trp, one-Trp, and Trp-less variants, in combination with this displacement technique, it was possible to demonstrate that Trp-86 and Trp-152 are involved in the fluorescence quenching associated with the binding of chloramphenicol, most likely via nonradiative energy transfer from these residues to the bound substrate. Trp-152 is mainly responsible for the fluorescence enhancement accompanying the binding of acetyl-CoA (and CoA) through proximity effects and solvent exclusion on substrate association.

Chloramphenicol acetyltransferase (CAT;[1] EC 2.3.1.28) catalyzes acetyl transfer from acetyl-CoA to the 3-hydroxyl of chloramphenicol, an inhibitor of the peptidyltransferase activity of prokaryotic ribosomes (Shaw, 1967). The product of the reaction, 3-acetylchloramphenicol, fails to bind to bacterial ribosomes (Shaw & Unowsky, 1968) and is thus devoid of antibiotic activity. Genes specifying CAT are the most common determinants of microbial resistance to chlor-

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[1] Abbreviations: CAT, chloramphenicol acetyltransferase; CAT_{III}, type III variant of CAT; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); *p*-cyano-CM, D-*threo*-1-(4-cyanophenyl)-2-(dichloroacetamido)-1,3-propanediol; *p*-iodo-CM, D-*threo*-1-(4-iodophenyl)-2-(dichloroacetamido)-1,3-propanediol; TSE buffer, 50 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl and 0.1 mM EDTA.

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