Nucleotide sequences of genes encoding the type II chloramphenicol acetyltransferases of *Escherichia coli* and *Haemophilus influenzae*, which are sensitive to inhibition by thiol-reactive reagents

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Sensitivity of enzymes to inhibition by thiol-reactive reagents is often presented as evidence for the possible involvement of cysteine residues in substrate binding and catalysis or to highlight possible important differences in structure and mechanism between closely related enzymes. The primary phenotypic distinction between the enterobacterial type II chloramphenicol acetyltransferase (CAT_{II}; typified by the enzyme encoded by the *incW* transmissible plasmid pSa) and the CAT_I and CAT_{III} variants is the greatly enhanced susceptibility of CAT_{II} to inactivation by thiol-specific modifying reagents. Determination of the nucleotide sequence of the gene, *cat_{II}*, present on pSa and that of a related determinant, *cat_{IIIH}*, isolated from *Haemophilus influenzae* indicates that sensitivity to such reagents cannot be due to the presence of additional reactive cysteine residues in CAT_{II}. Comparative analysis of the inactivation of CAT_{II} and CAT_{III} by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 4,4'-dithiodipyridine (DTDP) and methyl methanethiosulphonate (MMTS) suggests that (i) inactivation occurs as a result of chemical modification of the same residue (Cys-31) in each enzyme, (ii) reagents that inactivate via a pseudo-first-order process (DTNB and DTDP) appear to bind with a greater affinity to CAT_{II}, and (iii) the intrinsic reactivity of Cys-31 in CAT_{II} greatly exceeds that of the corresponding residue in CAT_{III}. The results lead to the conclusion that a striking difference in chemical reactivity of a unique and conserved thiol group between closely related enzyme variants may not be easily explained even when a high-resolution tertiary structure is available for one of them. Plausible explanations include more favourable access of reagents to Cys-31 in CAT_{II} or an enhanced reactivity of its thiol group imposed by the side chains of residues that are not in immediate contact with it.

INTRODUCTION

Bacterial resistance to the antibiotic chloramphenicol, an inhibitor of the peptidyltransferase activity of prokaryotic ribosomes, is commonly conferred by the enzyme chloramphenicol acetyltransferase (CAT, EC 2.3.1.28) (Shaw, 1967). The enzyme catalyses transfer of the acetyl group of acetyl-CoA to the primary (C-3) hydroxy group of chloramphenicol, yielding 3-acetylchloramphenicol, which fails to bind to bacterial ribosomes. CAT variants have been isolated from numerous bacterial genera (Shaw, 1983), and in each case the enzyme is a trimer of identical 25000-*M_r* subunits (Leslie *et al.*, 1986; Harding *et al.*, 1987). The amino acid sequences of eight CAT variants are known and show a high degree of similarity to each other (Shaw & Leslie, 1989; Wren *et al.*, 1989; Murray *et al.*, 1989).

Three classes of CAT variant have been characterized among Gram-negative bacteria, designated types I, II and III (Shaw, 1983). The type I variant (CAT_I, encoded by transposon Tn9 and numerous naturally occurring plasmids) is well known since it was the first to be studied (Shaw, 1967; Shaw *et al.*, 1979) and by its use as a tool in the analysis of eukaryotic gene expression (Gorman *et al.*, 1982). The catalytically efficient type III enzyme (CAT_{III}) (Murray *et al.*, 1988) has, however, been the subject of more extensive analysis, including steady-state kinetics

(Kleanthous & Shaw, 1984), X-ray crystallography (Leslie *et al.*, 1988) and site-directed mutagenesis (Lewendon *et al.*, 1988, 1990). In contrast, remarkably little is known about the type II enzymes (CAT_{II}) other than that they are immunologically distinct from both CAT_I and CAT_{III} (Fitton *et al.*, 1978) and that they differ from the latter by virtue of their exquisite sensitivity to inactivation by thiol-specific modifying reagents (Gaffney *et al.*, 1978). The gene encoding CAT_{II} on plasmid pSa was used in the construction of the vector pSa152 (Tait *et al.*, 1983).

All CAT variants, with the exception of those isolated from staphylococci, exhibit a modest degree of susceptibility to inactivation by reagents such as 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). In each case the presence of chloramphenicol protects the enzyme from modification. The crystal structure of CAT_{III} complexed with chloramphenicol indicated that Cys-31 was the sole reasonable candidate for the target of DTNB inactivation (Fig. 1; Leslie *et al.*, 1988), a proposition that is supported by the observation that the staphylococcal CAT variants said to be 'insensitive' to thiol-reactive reagents contain threonine at this position. The hypothesis was proven by the substitution of Cys-31 in CAT_{III} by alanine, methionine, serine or threonine, by using site-directed mutagenesis (Lewendon & Shaw, 1990). Each enzyme substituted at residue 31 was completely resistant to inactivation due to thiol-group modification. Furthermore, it

Abbreviations used: CAT, chloramphenicol acetyltransferase (subscripts denote individual variants); *cat*, gene encoding CAT; Ap^r, ampicillin-resistance phenotype; Cm^r, chloramphenicol-resistance phenotype; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); DTDP, 4,4'-dithiodipyridine; MMTS, methyl methanethiosulphonate; TSE buffer, 50 mM-Tris/HCl buffer, pH 7.5, containing 100 mM-NaCl and 0.1 mM-EDTA.

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X53796 (CAT_{II}) and X53797 (CAT_{IIIH}).

Fig. 1. Stereoview of the residues forming the chloramphenicol-binding site in CAT_{III}

The substrate chloramphenicol is represented in **boldface**. Residue numbers preceded by * belong to an adjacent subunit, and large circles indicate ordered water molecules. Hydrogen bonds are indicated by broken lines. The residue *HIS195 is the essential base that functions in the abstraction of a proton from the primary hydroxy group of chloramphenicol during catalysis (Kleanthous *et al.*, 1985).

was also shown that 4,4'-dithiodipyridine (DTDP) is a better inhibitor of wild-type CAT_{III} than is DTNB and that the inactivation by both reagents is a pseudo-first-order process. The evidence for a preliminary binding step led to the proposition that the hydrophobic environment of Cys-31 within the chloramphenicol-binding site discriminates against the binding of any ionized reagent such as DTNB. Mutations that are known to destabilize the tertiary structure of CAT_{III} also result in enhanced susceptibility to inactivation by DTNB, probably as a result of increased conformational flexibility and/or changes in the relative hydrophobicity of the chloramphenicol-binding site (Lewendon *et al.*, 1988).

The present paper describes the cloning and nucleotide sequence analysis of the gene, *cat_{III}*, from the *IncW* plasmid pSa and the nucleotide sequence of a related determinant, *cat_{IIIH}*, from *Haemophilus influenzae* (Spies *et al.*, 1983). Comparative analysis of the kinetics of inactivation of CAT_{III} and CAT_{II} (purified from *Escherichia coli* expressing the cloned gene) by DTNB, DTDP and methyl methanethiolsulphonate (MMTS) has been used to characterize and rationalize the enhanced sensitivity of type II variants of CAT.

MATERIALS AND METHODS

Materials

Restriction endonucleases, T4 DNA ligase and DNA polymerase I (Klenow fragment) were purchased from Bethesda Research Laboratories or Pharmacia and utilized according to the suppliers' recommendations. Deoxynucleotide triphosphates, dideoxynucleotide triphosphates and CoA were obtained from Pharmacia, calf intestinal alkaline phosphatase was from Boehringer and [α -³⁵S]thio[dATP] was from Amersham International. Antibiotics, DTDP, DTNB and MMTS were purchased from Sigma Chemical Co. or Aldrich Chemical Co. All other reagents were of analytical grade. Acetyl-CoA was prepared by the method of Simon & Shemin (1953).

Plasmid and bacteriophage M13 DNA

Plasmids pSa, pBR327 and pUC4 and bacteriophages M13 mp18 and M13 mp19 have been described previously (Valentine,

1985; Soberon *et al.*, 1980; Vieira & Messing, 1982; Yanisch-Perron *et al.*, 1985). The *cat* determinant of *H. influenzae* plasmid p85098 (Spies *et al.*, 1983) was kindly provided as an approx. 3.5 kb *Bam*HI subclone in pBR322 by Dr. T. Spies, University of Hamburg, Hamburg, Germany.

Plasmid and M13 RF DNAs were isolated by the method of Birnboim & Doly (1979) and single-stranded M13 DNA according to published methods (Sanger *et al.*, 1980). Transformation of *E. coli* JM101 (Messing, 1979) and identification of M13 recombinants was carried out as described by Gronenborn & Messing (1978).

Antibiotic-resistance phenotypes conferred upon *E. coli* by recombinant plasmids were determined by growth in the presence of ampicillin (100 μ g/ml) and/or chloramphenicol (10 μ g/ml) as appropriate.

Sequencing

The Sanger chain-terminator protocol (Sanger *et al.*, 1977) with buffer gradient gels and [α -³⁵S]thio[dATP] (Biggin *et al.*, 1983) was employed throughout. Synthetic oligonucleotide primers were prepared with an Applied Biosystems model 380B DNA synthesizer by J. W. Keyte, University of Leicester.

Purification of CAT

CAT_{II} was purified from extracts of *E. coli* by affinity chromatography on chloramphenicol-Sepharose as described previously (Lewendon *et al.*, 1988). Enzyme purity was assessed by PAGE in the presence of SDS (Laemmli, 1970) and protein determination by the method of Lowry *et al.* (1951), with wild-type CAT_{III} as standard.

Assay of CAT activity

CAT activity was monitored spectrophotometrically at 25 °C in TSE buffer as described in the preceding paper (Lewendon & Shaw, 1990). One unit of enzyme activity corresponds to 1 μ mol of chloramphenicol acetylated/min. Chloramphenicol and acetyl-CoA concentrations were varied during steady-state kinetic analyses, where initial rates were measured and all assays were carried out in triplicate (Kleanthous & Shaw, 1984). Kinetic

parameters, derived from linear slope and intercept replots from manually drawn double-reciprocal plots, were the means of two or more determinations with each of two separate enzyme preparations.

Chemical modification of CAT

Time courses of inactivation by thiol-specific chemical reagents were determined as described in the preceding paper (Lewendon & Shaw, 1990). The final concentration of CAT_{II} in each incubation was 0.8–0.9 mg/ml, equivalent to a monomer concentration of 3.2–3.6 μ M. Concentrations of modifying reagents varied as follows: DTNB, 20–800 μ M; DTDP, 40–200 μ M; MMTS, 10–50 μ M. Subsamples were taken at appropriate time intervals and diluted in TSE buffer before assay.

RESULTS AND DISCUSSION

Cloning of *cat*_{II}

A 5.4 kb *Bam*HI–*Eco*RI DNA fragment of plasmid pSa that included the *cat*_{II} gene was inserted into pBR327 to produce the recombinant plasmid pSaCm1 (results not shown). DNA of plasmid pSaCm1 was partially restricted with *Sau*3AI and ligated to *Bam*HI-cleaved pUC4. Transformation of *E. coli* with the ligation products yielded several Ap^r and Cm^r transformants, one of which contained a recombinant plasmid (pSaCm2) that included an approx. 1.4 kb *Sau*3AI partial-digestion product that by chance had regenerated *Bam*HI sites at both ends. pSaCm2 was utilized as the DNA source for subcloning in M13 mp18 and M13 mp19 to determine the sequence of *cat*_{II} (Fig. 2). An approx. 1.7 kb *Eco*RI fragment including *cat*_{IIIH}, derived from plasmid p85098, was used in the determination of the nucleotide sequence of the *Haemophilus* gene.

Nucleotide sequence analysis

The nucleotide sequences of *cat*_{II} and *cat*_{IIIH} genes were determined by using the strategy outlined in Fig. 2. Nucleotide sequence data obtained from individual restriction fragments

subcloned in M13 mp18 or M13 mp19 were extended, where appropriate, by the use of synthetic oligonucleotide primers designed according to the derived sequence.

The nucleotide sequences of 930 bp of pSaCm2 DNA and 877 bp of p85098 DNA were determined from both DNA strands without ambiguities, and in each case a *cat* open reading frame encoding 213 amino acid residues is predicted (Fig. 3). The deduced *N*-terminal sequence (Met-Asn-Phe-Thr-Arg-Ile-Asp) agrees with that determined by Edman degradation of type II CAT purified from *E. coli* harbouring plasmid pSa (Fitton *et al.*, 1978). The open reading frames are identical at 196 of 213 residues (92%), and the region of nucleotide sequence identity extends into the 5' and 3' non-coding DNA so that 778 of 877 (89%) aligned nucleotide residues are common to both genes.

The hexanucleotide AGAAGG, an approximation of the consensus sequence for prokaryotic ribosome association (AGGAGG; Shine & Dalgarno, 1975), occurs 13 residues before the ATG initiation codon. Both genes contain nucleotide sequences within the 5' and 3' non-coding regions that are similar to the consensus motifs for the initiation and termination of transcription in *E. coli* (Pribnow, 1975; Siebenlist, 1979; Rosenberg & Court, 1975).

Alignment of the deduced CAT_{II} amino acid sequence encoded by pSa with those of CAT_I (Shaw *et al.*, 1979; Alton & Vapnek, 1979) and CAT_{III} (Murray *et al.*, 1988) reveals extensive similarity, 45% and 65% residue identity respectively, most notably in the region surrounding the essential histidine residue, His-195 (Fig. 4). Side chains of amino acid residues known to contribute to the chloramphenicol-binding site in CAT_{III} (Fig. 1) are retained in the type II variants with the exception of the following substitutions: Phe-24→Leu, Gln-92→Val, Val-162→Ile and Ile-172→Val. Residues that could contribute directly to CoA binding, transition-state stabilization and stabilization of the tertiary structure of CAT_{III} are also retained (Day *et al.*, 1988; Lewendon *et al.* 1988, 1990). Significantly, neither CAT_{II} nor CAT_{IIIH} contains additional cysteine residues that might account for their particular sensitivity to thiol-reactive reagents.

Fig. 2. Sequencing strategy for the determination of the nucleotide sequences of *cat*_{II} and *cat*_{IIIH}

The Figure shows partial restriction maps of the *Bam*HI fragment of plasmid pSaCm2 and the *Eco*RI fragment derived from plasmid p85098 indicating restriction fragments that were subcloned into M13 mp18 or M13 mp19 for sequence analysis. Arrows indicate the extent and direction of sequence data derived from individual restriction fragments. Broken arrows indicate direction and extent of sequence data derived by using synthetic oligonucleotide primers synthesized according to the derived sequence. Key: B, *Bam*HI; E, *Eco*RI; Pv, *Pvu*II; Rv, *Eco*RV; S, *Sau*3AI; Sm, *Sma*I.

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1  GGATCCGCTATGTGT TTGCGGATGATTGGC CGGAATAAATAAAGC CGGGCTTAATACAGA TTAAGCCCGTAT-AG
1                                     * **** * *
1                                     GAATTC GCGAGCCCGAATCTG

75  GT---ATTATTACTG AATACCAAACAGCTT ACGGAGGACGGGAATG TTACCCATTGAGACA ACCAGACTGCCTTCT
*                                     * *
22  ATATCATTATT-CTG --TACCAAGCGGCTT ACGGAGGACGGGATG CTGCCCATTTGAGACA ACCAGACTGCCTTCT

147 GATTATTAATATTTT TCACTATTAATCAGA AGGAATAAACC Met Asn Phe Thr Arg Ile Asp Leu Asn
ATG AAT TTT ACC CGG ATT GAC CTG AAT *
94 GATTATTAATATTTT TCGCTATTAATCAGA AGGAATAAACC ATG AAT TTT ACC AGA ATT GAT CTG AAC

10                                     20
Thr Trp Asn Arg Arg Glu His Phe Ala Leu Tyr Arg Gln Gln Ile Lys Cys Gly Phe Ser
214 ACC TGG AAT CGC AGG GAA CAC TTT GCC CTT TAT CGT CAG CAG ATT AAA TGC GGA TTC AGC
*                                     *
161 ACC TGG AAC CGC AGA GAA CAT TTT GCT CTT TAT CGT CAG CAG ATA AAA TGC GGA TTC AGC

30                                     40
Leu Thr Thr Lys Leu Asp Ile Thr Ala Leu Arg Thr Ala Leu Ala Glu Thr Gly Tyr Lys
274 CTG ACC ACC AAA CTC GAT ATT ACC GCT TTG CGT ACC GCA CTG GCG GAG ACA GGT TAT AAG
*                                     *
221 CTG ACC ACA AAA CTC GAT ATT ACA GCT TTT CGT ACC GCA CTG GCG GAA ACG GAT TAT AAA
Phe                                     Asp

50                                     60
Phe Tyr Pro Leu Met Ile Tyr Leu Ile Ser Arg Ala Val Asn Gln Phe Pro Glu Phe Arg
334 TTT TAT CCG CTG ATG ATT TAC CTG ATC TCC CGG GCT GTT AAT CAG TTT CCG GAG TTC CGG
*                                     *
281 TTT TAT CCG GTG ATG ATT TAT CTG ATC TCC CGG GTT GTT AAT CAG TTT CCG GAG TTC CGG
Val                                     Val

70                                     80
Met Ala Leu Lys Asp Asn Glu Leu Ile Tyr Trp Asp Gln Ser Asp Pro Val Phe Thr Val
394 ATG GCA CTG AAA GAC AAT GAA CTT ATT TAC TGG GAC CAG TCA GAC CCG GTC TTT ACT GTC
*                                     *
341 ATG GCA ATG AAA GAT AAT GCA CTG ATT TAC TGG GAT CAG ACC GAT CCT GTA TTT ACT GTT
Met Ala                                     Thr

90                                     100
Phe His Lys Glu Thr Glu Thr Phe Ser Ala Leu Ser Cys Arg Tyr Phe Pro Asp Leu Ser
454 TTT CAT AAA GAA ACC GAA ACA TTC TCT GCA CTG TCC TGC CGT TAT TGT CCG GAT ATC AGT
*                                     *
401 TTT CAT AAA GAG ACT GAA ACA TTT TCT GCG CTC TTC TGC CGT TAT TGT CCG GAT ATC AGT
Phe                                     Cys Ile

110                                     120
Glu Phe Met Ala Gly Tyr Asn Ala Val Thr Ala Glu Tyr Gln His Asp Thr Arg Leu Phe
514 GAG TTT ATG GCA GGT TAT AAT GCG GTA ACG GCA GAA TAT CAG CAT GAT ACC AGA TTG TTT
*                                     *
461 GAA TTT ATG GCG GGC TAT AAT GCG GTG ATG GCA GAA TAT CAG CAT AAT ACT GCA TTG TTC
Met                                     Asn Ala

130                                     140
Pro Gln Gly Asn Leu Pro Glu Asn His Leu Asn Ile Ser Ser Leu Pro Trp Val Ser Phe
574 CCG CAG GGA AAT TTA CCG GAG AAT CAC CTG AAT ATA TCA TCA TTA CCG TGG GTG AGT TTT
***
521 CCG CAG GGA GCG TTA CCA GAG AAC CAC CTG AAT ATA TCA TCA TTA CCC TGG GTG AGT TTT
Ala

150                                     160
Asp Gly Phe Asn Leu Asn Ile Thr Gly Asn Asp Asp Tyr Phe Ala Pro Val Phe Thr Met
634 GAC GGA TTT AAC CTG AAC ATC ACC GGA AAT GAT GAT TAT TTT GCC CCG GTT TTT ACG ATG
*                                     *
581 GAC GGA TTT AAC CTG AAT ATC ACC GGT AAT GAT GAT TAT TTT GCT CCG GTG TTT ACT ATG

170                                     180
Ala Lys Phe Gln Gln Glu Gly Asp Arg Val Leu Leu Pro Val Ser Val Gln Val His His
694 GCA AAG TTT CAG CAG GAA GGT GAG CGC GTA TTA TTA CCT GTT TCT GTA CAG GTT CAT CAT
*                                     *
641 GCG AAA TTT CAG CAG GAA GAT AAC CGC GTA TTA TTA CCT GTT TCT GTA CAG GTA CAT CAT
Asp Asn

190                                     200
Ala Val Cys Asp Gly Phe His Ala Ala Arg Phe Ile Asn Thr Leu Gln Leu Met Cys Asp
754 GCA GTA TGT GAT GGC TTT CAT GCA GCA CCG TTT ATT AAT ACA CTT CAG CTG ATG TGT GAT
*                                     *
701 GCC GTT TGT GAT GGC TTT CAT GCA GCC AGG TTT ATT AAT ACA CTT CAG ATG ATG TGT GAT
Met

210
Asn Ile Leu Lys ***
814 AAC ATA CTG AAA TAA ATTAATTAATT CTGTATTTAAGCCAC CGTATCCGGCAGAAT GGTGGCTTTTTTTTA
*                                     *
761 AAC ATA CTG AAA TAA GTTAATTAATA CTTTATTTAAGCCAC TGTATCCGGAAGAAT GGTGGCTTTTTTTTA
*                                     *
884 TATTTTAACCGTAAT CTGTAATTCGTTTC AGACTGGTTCAGGA TC
* *** * *
831 TCTTTTCGTCGTGAT GTGTAATCTCGTTTC AGAATGGTTCAGGA TC

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Fig. 3. Nucleotide sequences and predicted open reading frames of *cat_{II}* and *cat_{III}*

The Figure shows aligned nucleotide sequences of *cat* genes originally isolated from plasmids pSa (upper) and p85098 (lower). Asterisks (*) indicate non-conserved nucleotide residues. Potential transcriptional initiation and termination consensus sequences are underlined. The amino acid residues predicted by the *Haemophilus* determinant are only indicated at positions where they differ from the equivalent residues in the pSa sequence.

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TYPE III:  MNYTKFDVKNWVRREHFEFYRHRLLPCGFSLSKIDITTLKKSLLDSAYKFYPVMI
          ** * * * * * * * * * * * * * * * * * * * * * * * * * * *
TYPE II:   MNFTRIDLNTWNRREHFALYRQIKCGFSLTTKLDITALRTALAETGYKFYPLMI
          * * * * * * * * * * * * * * * * * * * * * * * * * * *
TYPE I:    MEKKITGYTTVDISQWHRKEHFEAFQSVACQCTYNQTVQLDITAFLLKTVKKNKHKFYPAFI

TYPE III:  YLIAQAVNQFDELIRMAIKDDELIVWDSVDPQFTVFHQETETFSALSCPYSSDIDQFMVNY
          *** * * * * * * * * * * * * * * * * * * * * * * * * * * *
TYPE II:   YLISRAVNQFPEFRMALKDNELIYNDQSDPVFTVFHKEETETFSALSCRYFPDLSEFMAGY
          * * * * * * * * * * * * * * * * * * * * * * * * * * *
TYPE I:    HILARLMNAHPEFRMAMKDGELVIWDSVHPCYTVFHEQTETFSLLWSEYHDDFRQLHIY

TYPE III:  LSVMERYKSDTKLFPQGVTPENHLNISALPWVNFDSFNLNVANFTDYFAPIIITMAKYQQE
          * * * * * * * * * * * * * * * * * * * * * * * * * * *
TYPE II:   NAVTAEQHDTRLFPQGNLPENHLNISLFWVSFDGFLNITGNDDYFAPVFTMAKFQQE
          * * * * * * * * * * * * * * * * * * * * * * * * * * *
TYPE I:    SQDVACYGDNLAYFPKGFENMFFVSANPWVSFTSFDLNVANMDNFFAPVFTMGKYYTQ

TYPE III:  GDRLLPLSVQVHHAVCDGPHVARFINRLQELCNSKLK
          *** * * * * * * * * * * * * * * * * * * * * * * * * * * *
TYPE II:   GDRVLLPVSQVHHAVCDGFHAARFINTLQLMCDNLLK
          *** * * * * * * * * * * * * * * * * * * * * * * * * * * *
TYPE I:    GDKVLMPLAIQVHHAVCDGPHVGRMLNELQQYCDEWQGGAG

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Fig. 4. Alignment of the amino acid sequences of the enterobacterial type I, type II (pSa) and type III variants of CAT

Asterisks (*) indicate identical amino acid residues.

Steady-state kinetic analysis

CAT_{II} was purified to homogeneity from *E. coli* JM101 containing plasmid pSaCm2 and its specific activity was determined to be 423 ± 15 units/mg (cf. 830 and 155 units/mg for CAT_{III} and CAT_I respectively; Murray *et al.*, 1988; A. Lewendon, unpublished work). Steady-state kinetic parameters for CAT_{II} are shown in Table 1.

Chemical modification of CAT_{II}

Kinetic parameters determined for the inactivation of CAT_{II} by DTNB, DTDP and MMTS are shown in Table 2. In common with CAT_{III}, inactivation by both DTNB and DTDP occurs in the absence of bound chloramphenicol and shows rate saturation with increasing concentration of inhibitor, whereas reaction with MMTS fails to do so, as expected for a bimolecular process without a preliminary binding step. CAT_{II} that has been modified with a large molar excess of MMTS still retains approx. 4% of initial activity and is insensitive to further modification by the reagent. The same level of residual activity was observed with MMTS-modified CAT_{III} (Lewendon & Shaw, 1990), supporting the proposition that Cys-31 is the modified residue leading to loss of activity in both cases.

Both DTNB and DTDP bind with greater affinity to CAT_{II} and inactivate it more rapidly. However, the use of k_i/K_{inact} values as a guide to productive binding suggests that DTDP is a 41-fold better inhibitor of CAT_{II} than is DTNB, a result that parallels the discrimination (48-fold) against the latter reagent observed with CAT_{III}. The greater affinity of CAT_{II} for the disulphide inhibitors may be a consequence of amino acid substitutions within the chloramphenicol-binding site. For example, inspection of the CAT_{III} structure suggests that the

Table 1. Kinetic parameters for CAT_{II}

Numbers in parentheses are the corresponding parameters (Lewendon *et al.*, 1988) determined for CAT_{III}.

k_{cat} (s ⁻¹)	K_m for chloramphenicol (μM)	K_m for acetyl-CoA (μM)
238 (599)	10.3 (11.6)	65 (93)

Table 2. Kinetics of inactivation of CAT_{II} by thiol-reactive reagents and comparison with the corresponding parameters of CAT_{III}

The apparent second-order rate constant, k_i/K_{inact} , is formally analogous to k_{cat}/K_m , the specificity constant for catalysis. Numbers in parentheses are the corresponding values determined for CAT_{III} (Lewendon & Shaw, 1990). Abbreviation: N.A., not applicable.

Reagent	Bimolecular rate constant (M ⁻¹ ·s ⁻¹)	Pseudo-first-order rate constant (k_i) (s ⁻¹)	K_{inact} (mM)	k_i/K_{inact} (M ⁻¹ ·s ⁻¹)
DTNB	N.A.	3.5×10^{-3} (2.7×10^{-4})	0.68 (4.5)	5.1 (0.06)
DTDP	N.A.	3.8×10^{-2} (1.8×10^{-3})	0.18 (0.62)	208.2 (2.9)
MMTS	240 (19.6)	N.A.	N.A.	N.A.

substitution of Phe-24 by leucine could improve the accessibility of either inhibitor to Cys-31. However, in the absence of detailed structural analysis of the binding of the inhibitors to the enzyme surface (of either variant), such a hypothesis remains highly speculative.

The bimolecular rate constant for the inactivation of CAT_{II} by MMTS ($240 \text{ M}^{-1} \cdot \text{s}^{-1}$) is 12-fold greater than that of the same reagent with CAT_{III}. The clear implication of this observation is that the intrinsic reactivity of Cys-31 is greater in the type II enzyme and that this constitutes the major component of the general sensitivity of CAT_{II} to thiol-group modification. The fact that Cys-31 is more reactive in CAT_{II} may reflect improved access of the reagents to the reactive thiol group or differing pK_a values for the thiol group in the two enzymes. The only residue providing a side chain that is in close proximity to that of Cys-31 in CAT_{III} that is substituted in CAT_{II} is Val-162, which is replaced by isoleucine. Introduction of the same amino acid replacement into CAT_{III}, by site-directed mutagenesis, however, does not result in increased sensitivity to thiol-reactive reagents (I. A. Murray, unpublished work). In principle, a difference between the two enzymes of 1 pH unit for the pK_a of Cys-31 could account for a 12-fold difference in the reaction rates of CAT_{II} and CAT_{III} with MMTS. Such a change in pK_a could result from amino acid substitutions either within the chloramphenicol-binding site or at remote sites within the protein (Russell & Fersht, 1987). The former can probably be discounted in this case, for two reasons. First, mutations that alter the hydrophobicity of the chloramphenicol-binding site in CAT_{III} are known to have a profound effect on substrate binding (Day, 1990; I. A. Murray, unpublished work), whereas the measured k_m values for chloramphenicol are very similar ($10.3 \mu\text{M}$ and $11.6 \mu\text{M}$ for CAT_{II} and CAT_{III} respectively). Furthermore, changes to the relative hydrophobicity of the binding site would be expected to effect binding of the anionic (DTNB) and uncharged (DTDP) reagents differentially, which is not evident when comparing CAT_{II} and CAT_{III}. Long-range electrostatic interactions are known to have dramatic effects upon the binding of CoA and CoA analogues by CAT_{III} (Day, 1990), and it is conceivable that the pK_a of Cys-31 in CAT_{II} is modulated in this manner. Direct determination of the pK_a of the thiol group of Cys-31 in the two enzymes, in combination with site-directed mutagenesis of CAT_{III}, should provide further insights into the molecular mechanisms underlying the enhanced reactivity of this residue in CAT_{II}.

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