Comparison of Chloramphenicol Acetyltransferase Variants in Staphylococci

PURIFICATION, INHIBITOR STUDIES AND N-TERMINAL SEQUENCES

By JOHN E. FITTON* and WILLIAM V. SHAW

Department of Biochemistry, University of Leicester, Leicester LE1 7RH, U.K.

(Received 21 July 1978)

Four electrophoretic variants of chloramphenicol acetyltransferase (types A, B, C and D) found in chloramphenicol-resistant staphylococci were purified by affinity chromatography. Michaelis constants and the kinetics of inactivation with a variety of reagents for the four variants are virtually identical. Their similar amino acid compositions and near identical N-terminal sequences suggest a high degree of overall sequence homology. The thiol-specific reagents 5,5'-dithiobis-(2-nitrobenzoic acid), 2-nitro-5-thiocyanobenzoic acid and 2,2'-dithiopyridine are without significant effect on enzyme activity, whereas 1-fluoro-2,4-dinitrobenzene, N-ethylmaleimide, *p*-chloromercuribenzoic acid, iodoacetamide, and, particularly, bromoacetyl-CoA and diethyl pyrocarbonate are potent inhibitors. Iodoacetate is not an inhibitor. The results of chemical modification studies on the four enzyme variants and the identification of 3-carboxymethylhistidine in acid hydrolysates of one variant (type C) after inactivation with iodoacetamide suggest that a unique histidine residue may be involved in the mechanism of catalysis.

The bacterial enzyme chloramphenicol acetyltransferase (EC 2.3.1.28) catalyses the *O*-acetylation of chloramphenicol by acetyl-CoA (Shaw, 1967). The overall reaction for the inactivation of the antibiotic is:

Chloramphenicol acetyltransferase has been found in many genera of bacteria and is frequently specified by plasmid-associated genes (Shaw, 1974). Much of the attention to date has been focused on the Enterobacteriaceae, wherein the three main enzyme variants are synthesized constitutively and have been shown to differ with respect to net charge of the native protein, apparent substrate affinities, sensitivity to thiol inhibitors and immunological reactivity (Foster & Shaw, 1973; Fitton *et al.*, 1978). One such enteric variant has been shown to be especially sensitive to inhibition by a thiol-specific reagent (Foster & Shaw, 1973) and previous studies have stressed the likelihood that essential thiol groups may be involved in the mechanism of catalysis (Zaidenzaig & Shaw, 1978). All chloramphenicol acetyltransferase variants studied in detail are tetrameric proteins composed of four identical subunits, each subunit having a mol.wt. in the range 23 000–25 000.

Rather less work has been done on chloramphenicol acetyltransferase variants found in Gram-positive organisms, where enzyme synthesis is induced by

chloramphenicol or closely related analogues. The four staphylococcal variants (A, B, C and D) which have been described previously and shown to have different native electrophoretic mobilities are immunologically cross-reactive with antiserum prepared against the type-C variant (Sands & Shaw, 1973), but are immunologically distinct from chloramphenicol acetyltransferase variants purified from Gram-negative organisms. Certain staphylococcal variants have been shown to undergo subunit hybridization *in vitro* (Sands & Shaw, 1973).

The studies described here were performed to extend the knowledge of structural and functional properties of chloramphenicol acetyltransferases from staphylococci and to confirm the view that the four variants are closely related in primary structure and enzymic mechanism.

Materials

Chemicals

Dithiothreitol, β -mercaptoethanol, chloramphenicol and diethyl pyrocarbonate were from Sigma Chemical Co., St. Louis, MO, U.S.A.; DL-threo-norpseudoephedrine hydrochloride (DL-threo-2-amino-1-phenylpropan-1-ol hydrochloride) was from Phase Separations, Queensferry, Clwyd, Wales, U.K.; Whatman chromatography paper, polyamide thin layer and alumina thin layer (containing fluorescent indicator) chromatography sheets were from BDH Chemicals, Poole, Dorset, U.K.; iodo[2-¹⁴C]-acetamide was from The Radiochemical Centre,

* Present address: Department of Biochemistry, University of Sheffield, Sheffield S10 2TN, U.K.

Amersham, Bucks., U.K.; methyl dichloroacetate and 2,2'-dithiopyridine were from Aldrich Chemical Co., Milwaukee, WI, U.S.A.; dimethylformamide, *N*-methylmorpholine, trifluoroacetic acid, triethylamine, phenylisothiocyanate, aminopropyl-glass (7.5 nm porosity), guanidine hydrochloride and 5,5'-dithiobis-(2-nitrobenzoic acid) were from Pierce Chemical Co., Rockford, IL, U.S.A.; methanol, dichloroethane and pyridine were from Rathburn Chemical Co., Walkerburn, Peebleshire, Scotland, U.K.; acetyl-CoA was from Boehringer Mannheim G.m.b.H., Mannheim, Germany; and *p*-phenylene di-isothiocyanate was from Eastman Chemicals, Rochester, NY, U.S.A. DL-*threo*-2-*N*-Dichloroacetamido-1-*p*-nitrophenylpropan-1-ol was synthesized from DL-*threo*-norpseudoephedrine hydrochloride (Rebstock *et al.*, 1949). Bromoacetyl-CoA was prepared by transacetylation through a thiophenol intermediate (Chase & Tubbs, 1969). 2-Nitro-5-thiocyanobenzoic acid was synthesized from 5,5'-dithiobis-(2-nitro-benzoic acid) (Degani & Patchornik, 1974). Methyl dichloroacetate and ethyl acetate were redistilled before use. *p*-Phenylene diisothiocyanate, iodoacetic acid and iodoacetamide were recrystallized.

Bacterial strains

The four electrophoretic variants of chloramphenicol acetyltransferase used in this study have been described previously (Sands & Shaw, 1973). The producing organism of the type-A variant was isolated from military personnel in Vietnam (Sands & Shaw, 1973). The type-B variant was purified from *Staphylococcus epidermidis*, strain 39 NC (Bentley *et al.*, 1970). The type-C variant was purified from *Staphylococcus aureus*, strain RN 1305, a chloramphenicol-resistant transductant of *S. aureus* strain 8325-4, which carries the plasmid pC221. Type-D enzyme was purified from a chloramphenicol-resistant isolate of *S. aureus* from Bogota, Colombia (Sands & Shaw, 1973).

All cultures were frequently subcultured on Penassay agar (Difco, Detroit, MI, U.S.A.) containing 50 µg of chloramphenicol/ml to maintain plasmid-rich stock cultures.

Methods

Chloramphenicol acetyltransferase assay and kinetic measurements

Chloramphenicol acetyltransferase activity was assayed by the spectrophotometric method (Shaw, 1975). The concentration of 2-nitro-5-thiobenzoate produced was measured by absorbance at 412 nm ($\epsilon = 13600 \text{ M}^{-1} \cdot \text{cm}^{-1}$).

The rates of inactivation by the various reagents were calculated graphically from the equation

$\ln E/E_0 = -kt$, where E/E_0 is the fraction of the initial enzyme activity remaining after treatment with the reagent for time t (Colman, 1969).

Enzyme purification

Bacterial strains were grown on a rotary shaker at 37°C in 2-litre flasks containing 1.25 litre of Penassay broth and the 'gratuitous' inducer DL-*threo*-2-*N*-dichloroacetamido-1-*p*-nitrophenylpropan-1-ol (Sands & Shaw, 1973) at a concentration of 0.02 mM. Cells were harvested in early stationary phase by centrifugation at 20000g for 20 min at 4°C. The cell paste was washed with 50 mM-Tris/HCl, pH 7.8, containing 0.05 mM-chloramphenicol and resuspended in 500 ml of the same buffer for cell disruption by lyso-staphin (5 units/ml) and deoxyribonuclease (50 µg/ml) at 37°C. Lysis was complete in 1.5 h as judged by the decrease in A_{660} . The crude lysates were clarified by centrifugation at 20000g for 20 min. Since enzyme variants A and C were found to be relatively resistant to heat denaturation, the crude extracts were heated to 70°C for 10 min followed by centrifugation to remove precipitated protein. The final purification step (the only step in the case of the B and D variants) involved the use of affinity resins prepared as described previously (Zaidenzaig & Shaw, 1976). Crude extracts were loaded on to affinity columns (2.5 cm × 20 cm) of the required ligand substitution and washed with 50 mM-Tris/HCl, pH 7.8, and 50 mM-Tris/HCl, pH 7.8, containing 0.3 or 0.6 M-NaCl as required. The pure enzymes were eluted by 50 mM-Tris/HCl, pH 7.8, containing 5 mM-chloramphenicol and 0.3 or 0.6 M-NaCl.

Sodium dodecyl sulphate/polyacrylamide and agarose gels

Sodium dodecyl sulphate/11.5% polyacrylamide gel slab electrophoresis was performed using the buffer system of Laemmli (1970). Agarose gels of native proteins were run in 65 mM-sodium barbital buffer, pH 8.6, at 90 V for 1 h. Proteins were detected by staining with Coomassie Blue.

Chemical modification of enzyme variants

All modification experiments were performed in 50 mM-Tris/HCl, pH 7.8, at 37°C, except those with diethylpyrocarbonate where the conditions were 50 mM-potassium phosphate buffer, pH 6.0, at 0°C. Enzymes eluted from affinity columns were used in all cases after prior dialysis against 50 mM-Tris/HCl, pH 7.8, and the final protein concentrations were 0.2 mg/ml. The reagents and final concentrations in the incubations were as follows: 0.1 mM-diethyl pyrocarbonate; 1.0 mM-1-fluoro-2,4-dinitrobenzene; 1.0 mM-*N*-ethylmaleimide; 1.0 mM-5,5'-dithiobis-(2-

nitrobenzoic acid); 1.0mM-2-nitro-5-thiocyanobenzoic acid; 1.0mM-2,2'-dithiopyridine; 1.0mM-*p*-chloromercuribenzoic acid; 0.05mM-bromoacetyl-CoA; 5.0mM-iodoacetic acid; 5.0mM-iodoacetamide. Substrate protection was investigated by prior inclusion of substrate (1.0mM) with the enzymes for 10min at 20°C.

Photo-oxidation of type-C chloramphenicol acetyltransferase

Photo-oxidation was performed at 6°C with a 150W Crompton spot-light at a distance of 10cm from a water-jacketed conical tube containing the buffered enzyme solution (50mM-K₂HPO₄/KH₂PO₄, pH 5.5–7.0, or 50mM-Tris/HCl buffer, pH 7.3–9.0) and photosensitive dye (Methylene Blue). The final concentration of dye (3 µM) was approximately equal to that of protein monomers. Samples were withdrawn and immediately diluted 10-fold with 100mM-Tris/HCl, pH 8.0, in small tubes covered with aluminium foil.

Control experiments were performed under identical conditions in the dark and substrate protection was investigated by prior incubation of enzyme with substrate (1.0mM) for 10min at 20°C.

Amino acid analyses

Native and performic acid-oxidized proteins were hydrolysed in 6M-HCl containing phenol (0.01 mM) for 24, 48 and 72h at 105°C in evacuated borosilicate tubes. Recoveries were normalized by multiplying by a factor such that the sums of all the amino acids except threonine, serine, valine and isoleucine became equal in each analysis. Serine and threonine analyses were zero-time extrapolations, whereas those for valine and isoleucine were taken from extrapolations to infinite hydrolysis times. Tryptophan was determined after hydrolysis with 4M-methanesulphonic acid for 48h at 105°C. All analyses were performed on a single column amino acid analyser obtained from Locarte Scientific Instruments, London E.C.3, U.K.

N-Terminal sequence determinations

All *N*-terminal sequences were determined on an Anachem APS 2400 solid-phase sequencer. Phenylthiohydantoin amino acids were identified by t.l.c. with the exception of histidine and arginine, which were identified by amino acid analysis after hydrolysis with 55% HI (Bridgen *et al.*, 1975). Where no amino acid was identified by either of these methods on unmodified, carboxymethylated or performic acid-oxidized protein the residue was assumed to be lysine, which remains coupled to the solid-phase support using the di-isothiocyanate coupling procedure. *N*-

Terminal residues were identified by dansylation of the *N*-terminal amino acid (Gray, 1972). Freeze-dried salt-free protein samples were coupled to the isothiocyanate derivative of aminopropyl-glass by dissolving the protein (4mg) in 0.5ml of 10% (v/v) triethylamine/trifluoroacetic acid, pH 9.5, saturated with guanidine hydrochloride and stirred at 45°C. Samples (50mg) of the glass derivative were added at 5 min intervals. The reaction vessel was flushed with N₂ after each addition until a total of 200mg of glass had been added. The coupling reaction was left to proceed for a further 30min. Excess isothiocyanate groups were blocked by the addition of 0.1ml of ethanolamine and stirring continued at 45°C for a further 30min. The resulting glass derivative was washed with 2 × 5ml of methanol and dried under vacuum.

Results and Discussion

Protein purification

Although three of the four staphylococcal enzymes have been purified previously (Sands & Shaw, 1973), the methods originally used (salt precipitation, ion-exchange chromatography and gel filtration) were labour-intensive, subject to variation and resulted in overall yields of less than 20%. In contrast, the use of affinity chromatography permits a rapid and high-yield purification (Table 1). Considerable differences were observed between the four staphylococcal variants as regards the extent of ligand substitution of the affinity resin and salt concentration of the eluting buffer required to bind and elute the enzymes (Table 5). This elution pattern was not due to ionic concentration or composition of the cell-free extracts since purified enzymes behaved in a similar manner after prior dialysis against 50mM-Tris/HCl buffer, pH 7.8. As previously noted with chloramphenicol acetyltransferase variants of Gram-negative origin (Zaidenzaig & Shaw, 1976), up to 20% of the enzyme activity may be eluted from such affinity columns during the loading operation in low-salt buffer (50mM-Tris/HCl, pH 7.8), especially when loading is carried out at high flow rates. In such instances, the enzyme appearing in the earliest fractions can be recycled to increase overall final yields of pure enzyme to 90%. Without recycling, yields of 55–87% have been obtained (Table 1).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the purified proteins shows single bands which co-migrate with the so-called type-I chloramphenicol acetyltransferase purified from *Escherichia coli* (plasmid JR66b) which, from its amino acid sequence (W. V. Shaw, D. Burleigh, A. Dell, H. Morris & B. Hartley, unpublished work), has a mol.wt. of 23 500. Electrophoresis of native proteins on agarose gels (Fig. 1) shows clearly the variation in

Table 1. *Purification of staphylococcal chloramphenicol acetyltransferase variants*
Each preparation was started with the net weight of cells shown.

Type	Starting weight of cells (g)	Purification step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg of protein)	Yield (%)
A	46	Cell-free extract	430	4480	10148	0.44	100
		Heat step	400	4000	2720	1.47	89
		Affinity column	180	2400	46.8	51	55
B	24	Cell-free extract	260	1644	2678	0.61	100
		Affinity column	320	1440	55	26	87
C	37	Cell-free extract	400	635	5200	0.12	100
		Heat step	380	600	2500	0.24	95
		Affinity column	72	420	14	30	67
D	42	Cell-free extract	435	1535	1226	0.13	100
		Affinity column	66	1010	26.4	38	66

Table 2. *Affinity chromatography of staphylococcal chloramphenicol acetyltransferase variants*

Sephacrose 4B was substituted with 6-aminohexanoic acid (10 μ equiv. carboxy groups/ml of gel). The ligand D-threo-2-amino-1-p-nitrophenylpropan-1,3-diol was coupled to the gel at the degree of substitution indicated using a water-soluble carbodi-imide (Zaidenzaig & Shaw, 1976). The degree of substitution was determined by titration of the gel with alkali before and after coupling the free amine of chloramphenicol to the hexanoyl-Sepharose.

Enzyme variant	Ligand substitution (μ equiv./ml of gel)	NaCl concentration of eluting buffer [50 mM-Tris/HCl (pH 7.8)/5 mM-chloramphenicol] (M)
A	7	0.6
B	9	0.6
C	2.5	0.3
D	9	0.6

net charge noted previously when the staphylococcal enzyme variants were characterized by electrophoresis on polyacrylamide supports (Sands & Shaw, 1973).

K_m determinations

Michaelis constants were determined using the spectrophotometric enzyme assay under saturating (100 μ M-chloramphenicol or 200 μ M-acetyl-CoA) second-substrate conditions (Table 3). In addition, double-reciprocal plots of velocity versus first-substrate concentration, where the second substrate was present at a concentration near to the *K_m* for that substrate (5 μ M for chloramphenicol and 50 μ M for acetyl-CoA) were also determined. Intersecting plots were produced for all four enzymes, the intercept being on the 1/[S] axis. These results are consistent with those obtained in an analogous study of a chlor-

Fig. 1. *Agarose-gel electrophoresis of staphylococcal chloramphenicol acetyltransferases*

Protein (1 μ g) was applied to each track of the agarose gel equilibrated with 65 mM-sodium barbital buffer, pH 8.6, and electrophoresed at 90 V for 1 h (7.8 V/cm). Proteins were detected by staining with Coomassie Blue. O denotes the origin.

amphenicol acetyltransferase variant from *E. coli* (Tanaka *et al.*, 1974). The kinetic evidence thus favours the view that the order of addition of substrates is sequential and therefore inconsistent with a mechanism involving an acyl-enzyme intermediate. Attempts to detect such an intermediate have in fact been uniformly unsuccessful (Zaidenzaig & Shaw, 1978; J. E. Fitton, unpublished work). As noted with chloramphenicol acetyltransferase from *E. coli* (Zaidenzaig & Shaw, 1978) the purified staphylococcal variants are capable of deacylating acetyl-CoA in the absence of the acyl acceptor, chloramphenicol. The specific activity for this reaction is, however, less than 0.1% of the rate of formation of reduced CoA in the presence of chloramphenicol.

Table 3. *Apparent Michaelis constants for staphylococcal variants A, B, C and D*
 K_m values are shown $\pm$ s.e.

Enzyme variants	Variable substrate	K_m (μ M)
A	Chloramphenicol	2.58 ± 0.16
	Acetyl-CoA	57.3 ± 2.5
B	Chloramphenicol	2.72 ± 0.22
	Acetyl-CoA	56.1 ± 1.9
C	Chloramphenicol	2.47 ± 0.17
	Acetyl-CoA	60.9 ± 3.5
D	Chloramphenicol	2.70 ± 0.17
	Acetyl-CoA	46.5 ± 1.6

Chemical modification of staphylococcal chloramphenicol acetyltransferases

All four enzyme variants gave identical rates of inactivation with the test reagents within the range of experimental error ($\pm 3\%$). Only the mean values are presented in Table 4. The reagents 5,5'-dithiobis-(2-nitrobenzoic acid), 2-nitro-5-thiocyanobenzoic acid and 2,2'-dithiopyridine, which react with thiol groups via disulphide exchange, gave rates of inactivation that were indistinguishable from control samples.

Iodoacetamide inactivated all four enzyme variants at similar rates, whereas its anionic analogue, iodoacetate, did not. This dichotomy between the effects of the iodoacetyl reagents is identical with the inactivation properties of the type-I enzyme variant from *E. coli* (Zaidenzaig & Shaw, 1978). Inactivation of the type-C enzyme variant at pH values of 5.5 to 7.8 indicated an apparent pK_a of 6.3 for the inactivation process. Samples of the type-C enzyme variant were inactivated with iodo[2- 14 C]acetamide such that only 10% of the original enzyme activity remained.

After extensive dialysis, 1.0 mol of 14 C label was found to be incorporated per mol of enzyme monomer. On hydrolysis with 6M-HCl, amidocarboxymethyl amino acid derivatives are hydrolysed and the carboxymethyl derivatives can be identified by amino acid analysis. No carboxymethylcysteine was detected on analysis of acid hydrolysates of iodoacetamide, inactivated type-C enzyme, whereas 0.7 mol of 3-carboxymethylhistidine per mol of enzyme monomer was found. Dansylation (Gray & Hartley, 1963) of acid hydrolysates of iodo[2- 14 C]acetamide-inactivated type-C enzyme followed by polyamide t.l.c. (Hartley, 1970) and radioautography gave a single 14 C-labelled amino acid derivative which cochromatographed with the derivatives of lysine, arginine and histidine. No radioactivity was associated with the dansyl-carboxymethylcysteine spot.

The presence of chloramphenicol provided 95% protection against inactivation of all four enzyme variants by iodoacetamide, whereas acetyl-CoA gave less than 5% protection against inactivation (Table 4). Diethylpyrocarbonate proved to be a very effective inactivating reagent. Biphasic semilogarithmic plots of the loss of enzyme activity were obtained and the results were analysed on the basis of two first-order reactions. The rate constants were 2.10 and 0.496 min^{-1} , the latter being equal to the rate of inactivation in the presence of the substrate chloramphenicol and is, therefore, presumed to be due to the modification of functional group(s) not directly involved in chloramphenicol binding or catalysis. Approximately 50% of the inhibited activity could be recovered after treatment with 0.5M-hydroxylamine at pH 7.0 for 45 min, a procedure known to remove ethoxyformyl groups from histidine residues, but not from lysine or arginine derivatives (Melchior & Fahney, 1970). Again, co-incubation with chlor-

Table 4. *Mean rate constants for the inactivation of the four staphylococcal chloramphenicol acetyltransferase variants*

With the exception of diethyl pyrocarbonate, where 50mM-potassium phosphate buffer, pH 6.0, was used at 0°C , all inactivation experiments were performed in 50mM-Tris/HCl, pH 7.8, at 37°C . Control samples were treated in an identical manner to the test samples without addition of the test reagent. The four enzyme variants gave identical rates of inactivation ($\pm 3\%$) and mean values only are presented.

Reagent added	Concentration (mM)	$10^2 \times$ Rate constant (min^{-1})	Protection by 1 mM-chloramphenicol (%)	Protection by 1 mM-acetyl-CoA (%)
Diethyl pyrocarbonate	0.1	210.3	> 75	< 5
Bromoacetyl-CoA	0.05	55.3	> 80	> 85
1-Fluoro-2,4-dinitrobenzene	1.0	11.7	> 95	< 5
N-Ethylmaleimide	1.0	2.1	> 95	< 5
Iodoacetamide	5.0	1.8	> 95	< 5
p-Chloromercuribenzoate	1.0	0.85	< 5	< 5
Iodoacetate	5.0	0.08	—	—
5,5'-Dithiobis-(2-nitrobenzoic acid)	1.0	0.07	—	—
2-Nitro-5-thiocyanobenzoic acid	1.0	0.07	—	—
2,2'-Dithiopyridine	1.0	0.07	—	—
None	—	0.07	—	—

amphenicol protected each enzyme from inactivation, whereas acetyl-CoA failed to do so. Similarly, 1-fluoro-2,4-dinitrobenzene and *N*-ethylmaleimide inactivated all four enzyme variants with substrate protection observed only in the case of chloramphenicol (Table 4).

The substrate analogue bromoacetyl-CoA was an effective inactivating reagent at low concentration (0.05 mM). In this case both substrates protected the enzymes from inactivation. *p*-Chloromercuribenzoate inactivated the enzymes when used at high concentration (1 mM), but substrate protection was not observed with either chloramphenicol or acetyl-CoA suggesting that *p*-chloromercuribenzoate is not acting directly at the active site.

Photo-oxidation of a type-C enzyme variant

The pH profile of photo-oxidation (Fig. 2) shows a progressive loss of activity with increase in pH, the results being consistent with the photo-oxidation of a group with a pK_a of 6.9. Such a pH profile is compatible with the photo-oxidation of one or more histidine residues. Inclusion of chloramphenicol (1 mM) afforded complete protection against loss of activity.

Amino acid analyses and N-terminal sequences

Amino acid compositions of the four enzyme variants (Table 5) are expressed as numbers of residues

per monomer of mol.wt. 23 500. Although minor differences in the results of amino acid analysis are apparent, the four enzyme variants have similar amino acid compositions.

The *N*-terminal primary structures of the four enzyme variants were determined on samples of un-

Fig. 2. pH-dependence of the rate of photo-oxidation of staphylococcal type-C chloramphenicol acetyltransferase. Photo-oxidation was performed at 6°C in 50 mM-potassium phosphate buffers, pH 5.5–7.0, and 50 mM-Tris/HCl buffers, pH 7.3–9.0. Concentration of photosensitive dye (Methylene Blue) was 3 μ M.

Table 5. Amino acid composition of staphylococcal chloramphenicol acetyltransferases

Protein samples were hydrolysed in 6M-HCl containing phenol (0.01 mM) for 24, 48 and 72 h at 105°C. Serine and threonine values are extrapolations to zero time of hydrolysis and valine and isoleucine values are extrapolations to infinite time of hydrolysis. Cysteine was determined after performic acid oxidation and tryptophan by hydrolysis with 4M-methanesulphonic acid for 48 h at 105°C. The results were calculated for a mol.wt. of 23 500. N.D., not determined.

Enzyme variant	Composition (residues/mol)			
	A	B	C	D
Cysteic acid	N.D.	N.D.	1.5	N.D.
Aspartic acid	32.4	33.7	28.7	30.4
Threonine	10.1	9.5	16.0	10.8
Serine	14.9	13.3	14.6	12.7
Glutamic acid	18.8	17.0	18.7	19.2
Proline	8.6	9.3	11.2	9.3
Glycine	9.8	8.6	7.7	6.6
Alanine	3.9	4.7	4.8	3.8
Valine	16.2	10.7	7.5	7.3
Methionine	1.0	2.5	4.1	4.9
Isoleucine	16.8	17.7	23.9	22.4
Leucine	14.2	14.7	12.2	13.2
Tyrosine	12.5	11.8	10.7	12.3
Phenylalanine	13.7	15.2	12.3	13.8
Histidine	3.7	4.6	4.5	4.8
Lysine	15.6	16.9	17.5	18.2
Arginine	4.0	4.6	2.1	2.9
Tryptophan	N.D.	N.D.	4.1	N.D.

	1	5	10	15
Type A	Thr-Phe-Asn-Ile-Ile- <u>Asn</u> -Leu-Glu- <u>Thr</u> -Trp-Asp-Arg-Lys-Glu-Tyr-Phe			
Type B	Thr-Phe-Asn-Ile-Ile- <u>Lys</u> -Leu-Glu- <u>Thr</u> -Trp-Asp-Arg-Lys-Glu-Tyr-Phe			
Type C	Thr-Phe-Asn-Ile-Ile- <u>Lys</u> -Leu-Glu- <u>Asn</u> -Trp-Asp-Arg-Lys-Glu-Tyr-Phe			
Type D	Thr-Phe-Asn-Ile-Ile- <u>Glu</u> -Leu-Glu- <u>Asn</u> -Trp-Asp-Arg-Lys-Glu-Tyr-Phe			

Fig. 3. *N*-Terminal sequences of staphylococcal chloramphenicol acetyltransferase variants A, B, C and D. *N*-Terminal residues were identified by dansylation. Sequential analysis was performed on an Anachem APS 2400 solid-phase sequencer after coupling the protein samples to the di-isothiocyanate derivative of aminopropyl-glass. Residues were identified by t.l.c. with the exception of arginine and histidine, which were identified by amino acid analysis after hydrolysis with 55% HI at 130°C for 24 h. Where no residue could be identified by either of these methods using native, performic acid-oxidized or carboxymethylated protein the residue was assumed to be lysine. Lysine residues in the type-C enzyme variant have been positively identified by dansyl-Edman sequencing of peptides derived from proteolytic digests of the protein (Fitton, 1977). Variable residues are underlined.

modified as well as carboxymethylated and performic acid-oxidized protein. Performic acid-oxidized proteins gave a greater 'background' on t.l.c. plates, probably due to acid cleavage of the polypeptide and were unsuitable for extended sequence determinations. Unmodified proteins gave the cleanest results, in spite of the possibility of cleavage at cysteine residues during the sequencing procedure (Konigsberg, 1967). The four *N*-terminal sequences (Fig. 3) are identical save for the variation noted at residues 6 (Asn, Lys and Glu) and 9 (Thr and Asn).

The results of the kinetic and inactivation studies described indicate the functional similarities among four electrophoretic variants of chloramphenicol acetyltransferase found in staphylococci. In addition the *N*-terminal primary sequences indicate extensive structural similarities although two positions (residues 6 and 9) can accommodate amino acid substitutions without appreciable alteration in catalytic properties.

In contrast with chloramphenicol acetyltransferase variants from *E. coli* (Foster & Shaw, 1973; Zaidenzaig & Shaw, 1978), there is no evidence for a reactive cysteine residue in the staphylococcal enzymes. Although the classical thiol reagents iodoacetamide and *N*-ethylmaleimide inactivate the staphylococcal enzymes, evidence has been presented to support the view that it is the imidazole group of a histidine residue which is reactive in these experiments. The pH profiles of photo-oxidation and iodoacetamide inactivation of the type-C variant, the results with diethyl pyrocarbonate, and the identification of 3-carboxymethylhistidine rather than carboxymethylcysteine after inactivation with iodoacetamide suggest that a histidine residue may be of importance in the

mechanism of catalysis. More recent work on the type-I enzyme variant from *E. coli* has shown the presence of both 3-carboxymethylhistidine and carboxymethylcysteine after inactivation with iodoacetamide (J. E. Fitton, unpublished work). It would appear, therefore, that at least one of the Gram-negative chloramphenicol acetyltransferase variants has both a reactive cysteine and a reactive histidine at, or near, the active site, whereas the staphylococcal enzyme variants possess only the reactive histidine residue.

The research described in this report was supported by a Research Project Grant from the Medical Research Council of Great Britain. J. E. F. is the holder of a Medical Research Council Training Fellowship.

References

- Bentley, D. W., Sands, L. C. & Shaw, W. V. (1970) *J. Bacteriol.* **104**, 1095-1105
- Bridgen, J., Graffeo, A., Karger, B. L. & Waterfield, M. (1975) *Instrumentation in Amino Acid Sequence Analysis*, pp. 111-146, Academic Press, London
- Chase, J. F. A. & Tubbs, P. K. (1969) *Biochem. J.* **111**, 225-235
- Colman, R. F. (1969) *Biochemistry* **8**, 888-898
- Degani, Y. & Patchornik, A. (1974) *Biochemistry* **13**, 1-11
- Fitton, J. E. (1977) Ph.D. Thesis, University of Leicester
- Fitton, J. E., Packman, L. C., Harford, S., Zaidenzaig, Y. & Shaw, W. V. (1978) in *Microbiology - 1978* (Schlesinger, D., ed.), pp. 249-252, American Society of Microbiologists, Washington, DC
- Foster, T. J. & Shaw, W. V. (1973) *Antimicrob. Ag. Chemother.* **3**, 99-104

- Gray, W. R. (1972) *Methods Enzymol.* **25**, 121–138
- Gray, W. R. & Hartley, B. S. (1963) *Biochem. J.* **89**, 379–380
- Hartley, B. S. (1970) *Biochem. J.* **119**, 805–822
- Konigsberg, W. (1967) *Methods Enzymol.* **11**, 461–469
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Melchior, W. B. & Fahney, D. E. (1970) *Biochemistry* **9**, 251–257
- Rebstock, M. C., Crooks, H. M., Controulis, J. & Bartz, Q. R. (1949) *J. Am. Chem. Soc.* **71**, 2458–2462
- Sands, L. C. & Shaw, W. V. (1973) *Antimicrob. Ag. Chemother.* **3**, 299–305
- Shaw, W. V. (1967) *J. Biol. Chem.* **242**, 687–693
- Shaw, W. V. (1974) *Biochem. Soc. Trans.* **2**, 834–836
- Shaw, W. V. (1975) *Methods Enzymol.* **43**, 737–755
- Tanaka, H., Izaki, K. & Takahashi, H. (1974) *J. Biochem. (Tokyo)* **76**, 1009–1019
- Zaidenzaig, Y. & Shaw, W. V. (1976) *FEBS Lett.* **62**, 266–271
- Zaidenzaig, Y. & Shaw, W. V. (1978) *Eur. J. Biochem.* **83**, 553–562