Identification of 'buried' lysine residues in two variants of chloramphenicol acetyltransferase specified by R-factors

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Two variants of chloramphenicol acetyltransferase which are specified by genes on plasmids found in Gram-negative bacteria were subjected to amidination with methyl acetimidate to determine the relative reactivity of surface lysine residues and to search for unreactive or 'buried' amino groups which might contribute to stabilization of the native tetramers. Representative examples of the type-I and type-III variants of chloramphenicol acetyltransferase were found to have one lysine residue each in the native state which appears to be inaccessible to methyl acetimidate. The uniquely unreactive residue of the type-I protein is lysine-136, whereas the lysine that is 'buried' in the type-III enzyme is provisionally assigned to residue 38 of the prototype sequence. It is suggested that the lysine residue in each case participates in the formation of an ion pair at the intersubunit interface and that the two amino groups in question occupy functionally equivalent positions in the quaternary structures of their respective enzyme variants. Lysine-136 of the type-I enzyme is also uniquely unavailable for modification by citraconic anhydride, a reagent used to disrupt the quaternary structure of the native enzyme. Contrary to expectation, exhaustive citraconylation fails to dissociate the tetramer, but does destroy catalytic activity. Removal of citraconyl groups from modified chloramphenicol acetyltransferase is accompanied by a full regain of catalytic activity. Analysis of the rate of hydrolysis of citraconyl groups from the modified tetramer by amidination of unblocked amino groups with methyl [^{14}C]acetamidate reveals differences in lability for several of the ten modified lysine residues. Although the unique stability of the quaternary structure of chloramphenicol acetyltransferase may be due to strong hydrophobic interactions, it is argued that lysine-136 may contribute to stability via the formation of an ion pair at the subunit interface.

Bacterial resistance to chloramphenicol has been shown, in many cases, to be mediated through the enzymic action of chloramphenicol acetyltransferase (EC 2.3.1.28) (Shaw, 1967). This tetrameric enzyme, comprising identical subunits, catalyses the *O*-acetylation of chloramphenicol, with acetyl-CoA as the acyl donor, according to the equation:

Acetylchloramphenicol is devoid of antibacterial activity (Shaw, 1967).

Abbreviations used: SDS, sodium dodecyl sulphate; Dns- or dansyl-, 5-dimethylaminonaphthalene-1-sulphonyl.

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Many variants of chloramphenicol acetyltransferase have been identified in several genera of bacteria. In all cases, evidence for plasmid linkage of the structural gene is firm or circumstantial (Fitton *et al.*, 1978). Each variant may be characterized by a number of physicochemical parameters (e.g. electrophoretic mobility or temperature lability), and those enzymes possessing related properties may be assigned to a number of groups. Chloramphenicol acetyltransferase variants specified by R-plasmids and purified from *Escherichia coli*, after transfer of the plasmid in each case from its original host, comprise three classes: types I and II (each of M_r 24 000) and type III (M_r 24 500). The staphylococcal variants (types A, B, C and D; M_r 24 000), although closely related to each other, are structurally distinct from the R-plasmid-related enzymes purified from *E. coli* (Fitton *et al.*, 1978; Zaidenzaig *et al.*, 1979). The M_r values just quoted were

determined by polyacrylamide-gel electrophoresis in the presence of 0.1% (w/v) SDS. Sequence information suggests that these values may be some 5% lower than the true M_r .

In view of diverse properties, an examination of the primary structures of several selected variants is important to establish the structural, functional and evolutionary relationships between each. The primary structure of a type-I chloramphenicol acetyltransferase, coded for by plasmid JR66b (Benevise & Davies, 1971), has been determined, and the sequence data available for the closely related chloramphenicol acetyltransferase specified by plasmid R429 have been shown to be identical with the prototype sequence, with only one exception: asparagine-162 is replaced by an alanine residue in the enzyme coded for by plasmid R429 (Shaw *et al.*, 1979).

Work is required to establish the extent of sequence homology between the R-plasmid-coded enzymes of *E. coli* and their staphylococcal counterparts, and preliminary data in the form of *N*-terminal sequences have been reported (Fitton *et al.*, 1978). Internal sequence homology has also been found with varying degrees of sequence conservation. Evidence has been presented for the location of a uniquely reactive histidine residue in the active site of the type-C enzyme isolated from *Staphylococcus aureus* harbouring plasmid pC₂₁₁ (Fitton & Shaw, 1979). The primary structure of this region is conserved in all chloramphenicol acetyltransferase variants studied to date. The possible role of cysteine residues in the type-I (plasmid-JR66b-specified) variant has been examined by Zaidenzaig & Shaw (1978).

Rather less is known about the importance of lysine residues in chloramphenicol acetyltransferase variants. The present paper describes experiments which were performed to gain information about the topographical homology between the two R-plasmid-related variants, chloramphenicol acetyltransferase_{R429} (type-I enzyme coded for by plasmid R429) and chloramphenicol acetyltransferase_{R387}, a type-III enzyme.

The technique used was that of extensively labelling the amino groups in the native enzyme with methyl acetimidate, followed by modification of 'inaccessible' amino groups with radiolabelled reagent under denaturing conditions (Lambert & Perham, 1977; Lambert *et al.*, 1977). A unique lysine residue per subunit in each variant was identified as being totally inaccessible to solvent in the native enzyme under the conditions employed. There was no sequence homology between the peptides containing the unique lysine residue from each enzyme. The significance of these findings is discussed in the light of the determined partial sequence data for chloramphenicol acetyltransferase

ase_{R429} (type I) and chloramphenicol acetyltransferase_{R387} (type III).

Treatment of the native type-I enzyme with citraconic anhydride modified 10 of the 11 lysine residues, but failed to dissociate the tetramer. The uniquely inaccessible lysine was deduced to be that which was unavailable for modification by methyl acetimidate. The 'buried' lysine residue may either participate in ion-pair formation at the subunit interface or be shielded from solvent within the tertiary structure of constituent monomers.

Materials and methods

Chemicals

Trypsin and chymotrypsin were purchased from BDH Chemicals, Poole, Dorset, U.K. Methyl acetimidate was synthesized by the procedure of Hunter & Ludwig (1962). Methyl [1-¹⁴C]acetimidate was synthesized as described by Bates *et al.* (1975). All other chemicals were of analytical grade or the best grade available commercially.

Bacterial strains and plasmids

Chloramphenicol acetyltransferase_{R429} was isolated from *E. coli* strain J53LP (R429). This strain is a derivative of J53(R429) (Shaw *et al.*, 1972), which was selected for increased antibiotic resistance by growth on nutrient medium containing ampicillin (10 mg/ml). Chloramphenicol acetyltransferase activity in this organism was 5–6-fold greater than that observed in the wild type (Packman, 1978). The presence of plasmid R429 confers resistance to tetracycline as well as to ampicillin and chloramphenicol. Chloramphenicol acetyltransferase_{R387} was isolated from *E. coli* strain J53 harbouring plasmid R387, which confers resistance to streptomycin as well as to chloramphenicol (Hedges, 1975).

Purification of chloramphenicol acetyltransferase variants

E. coli J53LP(R429) was grown at 37°C in a 400-litre aerated batch culture at the Pilot Plant, Department of Biochemistry, Imperial College of Science and Technology, London, under the supervision of Mr. G. Banks. The growth medium consisted of medium 'E' (Vogel & Bonner, 1956) supplemented with 1.5% (w/v) casamino acids and 0.2% (v/v) glycerol.

E. coli J53(R387) was grown in the same medium in 15-litre batches in shaker flasks at 37°C.

Cells were broken by extrusion through a small orifice at high pressure in the presence of 0.1 M-Tris/HCl, pH 7.8, containing 2-mercaptoethanol (0.1 mM), chloramphenicol (0.2 mM), phenylmethanesulphonyl fluoride (50 µg/ml) and deoxyribonuclease I (1.0 µg/ml). Clarified supernatant was then incubated at 60°C for 10 min, and quickly

cooled to 2°C. Precipitate was removed by centrifugation and the supernatant was stored in 500–1000 ml batches, at –20°C, until required.

Chloramphenicol acetyltransferase was routinely purified to homogeneity by affinity chromatography by using chloramphenicol base coupled through its free amino group to aminohexanoyl-Sepharose 4B, as described previously (Zaidenzaig & Shaw, 1976). Certain variants of chloramphenicol acetyltransferase require the preparation of affinity resins of appropriate ligand substitution, with respect both to chloramphenicol base and to aminohexanoic acid, in order to ensure the recovery of the variants in a pure form and in high yield.

In the experiments described below, the Sepharose 4B contained 10 µmol of aminohexanoyl groups/ml of settled gel, as determined by titration of the free carboxy groups before coupling the latter to the free amino group of chloramphenicol. The affinity supports used for the purification of chloramphenicol acetyltransferase variants were substituted with chloramphenicol base as follows: (a) 5.0 µmol/ml of settled gel for the R429 variant and (b) 5.9 µmol/ml for the enzyme specified by plasmid R387.

Crude extracts were dialysed overnight against 50 mM-Tris/HCl (pH 7.8) containing 0.1 mM-2-mercaptoethanol ('standard buffer') to remove excess intrinsic salt and chloramphenicol, both of which inhibit binding of the enzymes to the affinity resin. After assaying the amount of chloramphenicol acetyltransferase present, an amount of washed affinity resin was chosen such that 1.0 ml of settled resin was available for each 200 units of enzyme. The resin was added in a thick slurry in the standard buffer to the stirred crude extract at 4°C, and binding of the enzyme was monitored by removing samples of the mixture, sedimenting the resin by centrifugation, and assaying the supernatant. Binding had usually reached a maximum within 10 min.

The resin was poured into a column at 4°C and washed with 1.5 column volumes of the equilibrating buffer to remove unbound enzyme. In succeeding steps of the purification, the NaCl concentration in the buffer was 0.3 M for chloramphenicol acetyltransferase_{R387} and 0.6 M for chloramphenicol acetyltransferase_{R429}. The resin was washed with standard buffer containing NaCl, and the column effluent was monitored for protein by its $A_{280}^{1\text{cm}}$ against the appropriate buffer/NaCl blank. When the absorbance had fallen to less than 0.02, elution of chloramphenicol acetyltransferase was performed with standard buffer containing 50 mM-NaCl and 5 mM-chloramphenicol. Chloramphenicol-eluted chloramphenicol acetyltransferase (90–100% yield) was dialysed against standard buffer containing 0.2 mM-chloramphenicol at 4°C. The protein was assayed for purity by polyacryl-

amide-gel electrophoresis under native and denaturing conditions and found to be homogeneous. The specific activity of each chloramphenicol acetyltransferase variant was determined at saturating substrate concentrations and found to be 195 units/mg (R429-specified variant) and 570 units/mg (R387-specified variant). Enzyme was stored in batches at –20°C.

Assay of chloramphenicol acetyltransferase

The enzymic acetylation of chloramphenicol was determined by the spectrophotometric assay (Shaw, 1975). The reaction was followed at 412 nm with a Pye-Unicam SP. 1800 dual-beam recording spectrophotometer. One enzyme unit is defined as that amount of enzyme which catalyses the production of 1 µmol of product/min at 37°C.

Protein determination

The protein content of crude extracts was determined by the method of Lowry *et al.* (1951). Homogeneous preparations of chloramphenicol acetyltransferase were determined by amino acid analysis with reference to the known compositions of the enzymes concerned (Packman, 1978).

Gel electrophoresis

Protein samples were run in the presence of 0.1% (w/v) SDS in the discontinuous buffer system of Laemmli (1970). Gels had the composition 12.5% T and 2.6% C, where T is concentration (w/v) of acrylamide and C is concentration of bisacrylamide as percentage (w/w) of acrylamide monomer (Hjerten, 1962). Electrophoresis of protein samples under native conditions used the same buffer system in the absence of SDS and sample buffer was replaced by 50 mM-Tris/HCl, pH 7.8, containing 0.1 mM-2-mercaptoethanol. All gels were stained overnight at room temperature in a solution of 0.0125% (w/v) Coomassie Brilliant Blue R dissolved in 40% (v/v) methanol and 7% (v/v) acetic acid. Destaining took place by diffusion in 7% (v/v) acetic acid.

Amino acid analysis

Protein and peptide samples were hydrolysed for 24 h *in vacuo* in the presence of 6 M-HCl containing 10 µM-phenol, at 110°C. The amino acid composition of hydrolysates was determined using a single-column Locarte amino acid analyser, operating with sodium citrate buffers (Spackman *et al.*, 1958).

Amidation of native chloramphenicol acetyltransferase

Native protein was fully equilibrated with freshly prepared buffer (0.2 M-triethanolamine/HCl, pH 8.5, containing 1 mM-2-mercaptoethanol) either by extensive dialysis at 4°C or by gel filtration at room temperature with Sephadex G-10. The protein

concentration was adjusted as necessary with the same buffer.

A stock solution (1.0M) of methyl acetimidate was prepared immediately before use by neutralizing the hydrochloride with 1 equivalent of NaOH and adding triethanolamine/HCl (pH 8.5) to a final concentration of 0.2M. Within 5 min of preparation, stock solution of imidoester was diluted into the protein-containing sample to a final imidoester concentration of 50mM. Amidination proceeded at room temperature for 2–3 h as described in the Results section, after which time a second addition of fresh reagent was made. As the rise in pH during the course of the reaction did not affect enzyme stability, no adjustments in pH were made during the course of the reaction.

Samples (10 μ l) were removed from the reaction mixture at timed intervals, and diluted into standard buffer and assayed for enzyme activity. Larger samples (100 μ l) were similarly removed and the amidination process in each was arrested by addition to an equal volume of 0.5M-ammonium acetate, pH 5.0. The samples were dialysed against 3 litres of distilled water and freeze-dried from aq. 10% (v/v) triethylamine to ensure complete removal of ammonia. When amidination was complete, the reaction was quenched by addition of 0.5M-ammonium acetate, pH 5.0, to a final concentration of 0.2M.

The fully amidinated protein was desalted by gel filtration on a Sephadex G-10 column (17cm $\times$ 1.5cm diameter) equilibrated with 0.2M-triethanolamine/HCl buffer, pH 8.5, containing 1mM-2-mercaptoethanol. Subsequent filtration on a Sephadex G-75 column (20cm $\times$ 1cm diameter) in the same buffer ensured the isolation of all tetrameric protein from soluble, denatured material of smaller molecular size.

Under the amidination conditions used, no cross-linking of chloramphenicol acetyltransferase, by *N*-alkylimidate formation (Brown & Kent, 1975), was observed, as judged by SDS/polyacrylamide-gel electrophoresis.

Amidination of denatured chloramphenicol acetyltransferase with methyl [1- 14 C]acetimidate

Chloramphenicol acetyltransferase was denatured, reduced and carboxymethylated as previously described (Zaidenzaig & Shaw, 1978). The enzyme was then dissolved in 0.2M-triethanolamine/HCl buffer, pH 8.5, containing 1mM-2-mercaptoethanol and 8m-guanidinium chloride, and was amidinated with 0.1M-methyl [1- 14 C]acetimidate. After 2h, a second addition of fresh reagent was made and the reaction continued for a further 2h. The reaction was quenched with an equal volume of 0.5M-ammonium acetate, pH 5.0, and the protein sample was exhaustively dialysed against 50mM-

NH₄HCO₃ and then freeze-dried. Complete amidination under these conditions was demonstrated by extrapolation of the *N*⁶-amidinolysine content of protein samples, hydrolysed for 24, 48 and 72h, back to zero time of hydrolysis. For chloramphenicol acetyltransferase_{R429} an extrapolated value of 11.3mol of *N*⁶-amidinolysine/mol of enzyme subunit was obtained. This is in precise agreement with the lysine content of the enzyme (11.3mol/mol of subunit) obtained by amino acid analysis (Packman, 1978). *N*⁶-Amidinolysine, which was eluted between ammonia and arginine on the amino acid analyser, was assumed to have the colour factor of arginine (Reynolds, 1968; Plapp & Kim, 1974) and, under the hydrolysis conditions employed, had a half-life of 68h. Radioactivity was determined by liquid-scintillation counting in a Packard Tri-Carb liquid-scintillation counter (model 3385) with Bray's fluid as scintillant (Bray, 1960). From these data, and the assumption that the *N*-terminus of the protein was uniformly labelled, the specific radioactivity of methyl [1- 14 C]acetimidate was determined as 0.077 μ Ci/ μ mol (170d.p.m./nmol).

Modification of chloramphenicol acetyltransferase_{R429} with citraconic anhydride

Chloramphenicol acetyltransferase (20mg) was equilibrated at room temperature with 0.2M-triethanolamine/HCl buffer, pH 8.5, containing 2-mercaptoethanol (1mM), by gel filtration on Sephadex G-10. Citraconylation was achieved by addition of 20 μ l of the anhydride (55mM final concn.) to 4ml of the stirred protein solution (5mg/ml) at 20°C. The pH of the solution was maintained at 8–8.5 by addition of small portions of 5M-NaOH in the same buffer.

Samples (10 μ l) were removed at timed intervals and diluted into 1.0ml of 50mM-Tris/HCl buffer (pH 7.8) containing 2-mercaptoethanol (0.1mM), before assay of enzyme activity. Larger samples (20 μ l) were removed and diluted into 100 μ l of 20mM-Tris/HCl buffer (pH 7.8) containing 2-mercaptoethanol (0.1mM), for electrophoresis on polyacrylamide gels under non-denaturing conditions. After 160min of incubation, another addition (20 μ l) of citraconic anhydride was made. Subsequent observations suggested that the second addition of anhydride was unnecessary; complete modification of amino groups is afforded by a single treatment with the reagent.

After 240min of incubation, citraconyl-chloramphenicol acetyltransferase_{R429} was equilibrated with 50mM-sodium phosphate buffer (pH 8.5) containing 2-mercaptoethanol (1mM) and chloramphenicol (0.2mM) by gel filtration on Sephadex G-75, from which the tetrameric enzyme is excluded. Less than

15% of the applied sample was eluted at or near the V_t (total volume) of the column, as judged by the A_{280} profile, suggesting the presence of only small amounts of the monomeric species. A sample of citraconyl-chloramphenicol acetyltransferase_{R429} (1 mg) was removed for electrophoretic analysis and for radioamidation under denaturing conditions (see below).

Removal of citraconyl groups from modified chloramphenicol acetyltransferase_{R429}

Citraconyl-chloramphenicol acetyltransferase_{R429} was concentrated to a volume of 2 ml in an Amicon B-15 Macrosolute Concentrator, and stored overnight at 4°C. The solution was diluted with 0.2 M-sodium phosphate buffer (pH 6.0) to a final enzyme concentration of 1 mg/ml, and incubated at 37°C in a sealed vial.

At timed intervals over a period of 3 days, the mixture was assayed for regain of enzyme activity and 1 ml samples were removed for further study. Each sample was immediately mixed with 1.5 ml of 1 M-NH₄HCO₃ (final pH 8.2), to arrest the hydrolysis of citraconyl-lysine, and stored at 0°C. After about 2 h of incubation at pH 6, precipitation of protein became visually evident in the regeneration mixture. Samples removed after this time were therefore centrifuged after dilution with NH₄HCO₃ and the precipitate was discarded. The samples of soluble enzyme were treated thereafter in similar fashion to a sample of fully citraconylated enzyme and a sample of untreated (native) enzyme.

All samples were dialysed at 4°C against 50 mM-NH₄HCO₃ buffer (pH 7.8) containing 2-mercaptoethanol (0.1 mM), and a 100 μ l portion of each was analysed by gel electrophoresis under denaturing conditions.

Measurement of the free lysine content of chloramphenicol acetyltransferase

The estimation of unlabelled amino groups in chloramphenicol acetyltransferase was not possible by the trinitrobenzenesulphonic acid assay (Habeeb, 1966), as precipitation of the enzyme occurred on addition of SDS to a final concentration of 2.5% (w/v), as is required for this procedure. Solubilization of chloramphenicol acetyltransferase by warming was unrewarding, as the trinitrobenzenesulphonic acid assay was found to be sensitive to changes in temperature (L. C. Packman, unpublished work).

Radioamidation of protein samples by methyl [1-¹⁴C]acetamidate, under denaturing conditions, was therefore used exclusively to determine the free amine content (Lambert & Perham, 1977) from a knowledge of the lysine content of each enzyme variant.

Proteolytic digestion

Enzymic digestion of protein samples was performed in 50 mM-NH₄HCO₃ with an enzyme/substrate ratio of 1:100 (w/w). Denatured protein, which was insoluble in this medium, was agitated to form an even suspension by using an ultrasonic disintegrator at maximum output for 15–20 s. Digestion proceeded at 37°C, with constant stirring, for 2–4 h, as stated in the text. Where necessary, a second addition of proteolytic enzyme was made after 2 h. Digestion was arrested and peptides were recovered by freeze-drying.

Paper electrophoresis

High-voltage paper electrophoresis of peptides at pH 6.5 and pH 2.1 was performed on Whatman no. 1 and 3MM chromatography paper with standard buffers (Brownlee, 1972). Mobilities of peptides at pH 6.5 and pH 2.1 were calculated relative to aspartic acid and serine respectively, by the method of Offord (1966). Peptides were eluted in 5% (v/v) acetic acid, and freeze-dried.

Thin-layer electrophoresis.

Analytical separation of peptides from enzymic digestion of ¹⁴C-labelled protein on a micro-scale was performed by the method of Bates *et al.* (1975), by using 10 cm $\times$ 10 cm POLYGRAM SIL G (Camlab) silica thin-layer plates and standard high-voltage paper-electrophoresis buffers. Each salt-free sample applied was derived from the proteolytic digestion of 1–5 nmol of protein. Samples were applied in 1–2 μ l of electrophoresis buffer, containing fluorescent markers (Dns-arginine and Dns-arginylarginine).

Electrophoresis was carried out at 350 V for 50 min, without cooling, in a Shandon (model Kohn U77) flat-bed electrophoresis apparatus. The edges of the thin-layer plate were connected to the electrode solutions by wicks of Whatman 3MM filter paper. After thorough drying, ascending chromatography was carried out, at right-angles to the direction of electrophoresis, in butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.; Waley & Watson, 1953) for 50 min.

Detection of peptides

Peptides on paper and thin-layer sheets were detected by staining with fluorescamine (10 μ g/ml, w/v, in acetone) (Udenfriend *et al.*, 1972), and, where appropriate, by radioautography.

Sequence analysis

Peptides were sequenced by the dansyl-Edman procedure (Hartley, 1970) and Dns-amino acids were identified by t.l.c. on polyamide sheets (Woods & Wang, 1967).

Results

Modification of native chloramphenicol acetyltransferase_{R429} with methyl acetimidate

Native chloramphenicol acetyltransferase_{R429} (16 mg, 5 mg/ml) was amidinated with 50 mM-methyl acetimidate as described in the Materials and methods section. Some precipitation of the enzyme occurred during the course of the reaction; this was monitored by increase in turbidity of the solution at 660 nm.

The modification of chloramphenicol acetyltransferase_{R429} was accompanied by a loss of enzyme activity, which stabilized at 40% of its initial value after 20 min of incubation. No further loss of activity accompanied a second addition of fresh reagent after 125 min of incubation (Fig. 1). After 205 min of incubation, the reaction was quenched and the precipitate (4 mg) was removed by centrifugation. On subsequent gel filtration of the supernatant, amidinated chloramphenicol acetyltransferase_{R429} was seen to have retained its quaternary structure. The modified enzyme had a specific activity of 70 units/mg and an electrophoretic mobility which was slightly less anionic than that of the untreated control. This increase in basicity may be related to

Fig. 1. Amidination of native chloramphenicol acetyltransferase_{R429} with methyl acetimidate

Native chloramphenicol acetyltransferase_{R429} was amidinated with 50 mM-methyl acetimidate as described in the text. A second addition of fresh reagent (final concn. 50 mM) was made at the point denoted by the arrow. Enzyme activity of amidinated enzyme (○); enzyme activity of untreated enzyme under the same incubation conditions (●); amino groups remaining unmodified by methyl acetimidate, assayed by subsequent treatment of partially modified protein with radiolabelled reagent under denaturing conditions (Δ); A_{660} of the reaction mixture (a measure of the amount of protein precipitation) (□); pH of the reaction mixture, continuously monitored by a pH-meter calibrated with triethanolamine/HCl buffer (·····).

the higher pK_a value of the amidine group compared with that of lysine (Ludwig & Hunter, 1967).

Since amidinated chloramphenicol acetyltransferase_{R429} still possessed an average of 1.28 free amino groups per subunit, further studies were directed at the identification of such groups within the primary structure of the enzyme. To this end, the analytical protein samples used to determine the number of free amino groups present in chloramphenicol acetyltransferase_{R429}, during amidination of the native enzyme, were digested with chymotrypsin (2 h) and then trypsin (2 h). The resulting peptides were 'mapped' on thin-layer silica sheets, and radioactive peptides were located after 10 days of radioautography (Fig. 2).

The peptide 'maps' confirmed the previous observation that the number of free amino groups (not amidinated by methyl acetimidate) in the native enzyme decreases rapidly over the first 40 min of the incubation and thereafter at a slower rate (Fig. 1). After 205 min, approx. 3 amino groups have not been amidinated, although the subsequent incorporation of ^{14}C from methyl [^{14}C]acetimidate at this point on denaturation is only 1.45 mol/mol of subunit. When the tetrameric material was isolated before labelling with methyl [^{14}C]acetimidate, the relative intensity of the neutral radioactive peptide(s) was markedly decreased. This observation suggests that samples (1)–(5), removed from the reaction mixture and processed without prior isolation of the tetrameric material, contained (precipitated or soluble) denatured enzyme and amino groups which were shielded from the reagent for reasons unrelated to the native structure. The results seen in 'map' 6 (Fig. 2) clearly show that it is likely that a unique lysine residue (in peptide IR-1) was inaccessible to amidination in the enzyme, and a possible second lysine residue (in peptide IR-2) was incompletely exposed or reactive.

Identification of the 'buried' lysine residues in chloramphenicol acetyltransferase_{R429}

Amidinated tetrameric chloramphenicol acetyltransferase_{R429} (7 mg) was radioamidinated in the presence of 8 M-guanidinium chloride, desalted and S-carboxymethylated.

After extensive dialysis, the protein was digested with chymotrypsin for 2 h and with trypsin for a further 2 h, in 50 mM- NH_4HCO_3 . The insoluble fraction, containing 5% of the radioactive material, was removed by centrifugation and discarded. The soluble peptides were fractionated by gel filtration on a Sephadex G-25 column (86 cm $\times$ 1.5 cm diameter), equilibrated and developed in the same buffer.

The fractions comprising the ^{14}C -containing peak were pooled and freeze-dried. Examination of this pool by electrophoresis and chromatography on thin-layer plates showed it to contain a minimum of

Fig. 2. Radioautographs of the two-dimensional 'maps' of peptides generated by proteolysis of amidinated chloramphenicol acetyltransferase_{R429}.

Native chloramphenicol acetyltransferase_{R429} was treated with methyl acetimidate as described in the Materials and methods section. Samples were removed from the reaction mixture after the time intervals shown, and these were *S*-carboxymethylated and treated with methyl [1-¹⁴C]acetimidate in the presence of 8M-guanidinium chloride. The samples, after determination of the incorporation of ¹⁴C into each, were digested with chymotrypsin and trypsin. The resulting peptides were analysed by electrophoresis and chromatography on silica gel. 'Map' no. 6 was derived from amidinated chloramphenicol acetyltransferase_{R429}, which was exclusively tetrameric. The average number of amino groups per subunit remaining unmodified in the native enzyme is shown at each time interval.

12 peptides, including peptides IR-1 and IR-2. The two radioactive peptides were purified on Whatman no. 1 paper by descending chromatography in butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.), followed by electrophoresis at pH 2.1. After electrophoresis in the second dimension, peptide IR-2 could be detected by radioautography only after being in contact with film for 2 weeks. Elution of the peptides and subsequent amino acid analysis showed there to be 44 nmol of peptide IR-1 recovered, but less than 4 nmol of peptide IR-2. The amino acid analysis of the latter peptide indicates that it is probably a proteolytic precursor of peptide IR-1, since it has a composition equivalent to that of peptide IR-1, plus three more amino acids (Table 1) known to be residues adjacent to those in peptide IR-1 in type-I variants of the enzyme.

Relating the sequence of peptide IR-1 to the known primary-structure data for chloramphenicol acetyltransferase variants specified by plasmids

R429 and JR66b (Shaw *et al.*, 1979), it is clear that the lysine residue inaccessible to solvent in chloramphenicol acetyltransferase_{R429} (and by inference in the plasmid-JR66b-specified variant as well) is lysine-136.

Modification of chloramphenicol acetyltransferase_{R387} with methyl acetimidate

Chloramphenicol acetyltransferase_{R387} (15 mg) was modified with 50 mM-methyl acetimidate in a final volume of 4 ml as described in the Materials and methods section. The overall amidination reaction had characteristics similar to those obtained for the amidination of chloramphenicol acetyltransferase_{R429} (Fig. 3). Enzyme activity was decreased to 30% of its initial value within 20 min and was unaffected by a second addition of imidoester after 132 min of incubation. In contrast with chloramphenicol acetyltransferase_{R429}, the enzyme specified by plasmid R387 showed only a

Table 1. Amino acid and sequence analyses of peptides IR-1 and IR-2 from chloramphenicol acetyltransferase_{R429} and peptides IP-1 and IP-2 from chloramphenicol acetyltransferase_{R387}

Radioactivity was associated with the release of the amino acid denoted by an asterisk, during the Edman degradation of each peptide. The radioactive amino acid, as the dansylated derivative, had chromatographic properties on polyamide thin-layer plates similar to those of *N*⁶- and *N*²-dansyl-lysine (Hartman & Wold, 1967). This was taken as sufficient evidence that the radioactive residue was lysine-amidine.

Amino acid	Composition of peptides (mol/mol) [†]			
	IR-1	IR-2	IP-1	IP-2
Asp	—	—	1.10	1.03
Thr	—	—	2.20	2.05
Ser	—	—	1.50	1.03
Pro	0.76	1.05	—	—
Gly	1.17	1.15	—	—
Ala	—	1.00	—	—
Ile	—	—	1.75	1.65
Leu	—	0.86	1.78	1.07
Tyr	—	0.70	—	—
Phe	1.50	2.20	—	—
Lys + Lys-amidine	1.05	1.00	1.00	1.04
¹⁴ C incorporation (mol/mol of peptide)	1.05	1.12	0.95	1.00

Pep- tide	Sequence	Yield
IR-1	Phe-Pro-Lys*-Gly-Phe	44 nmol
IP-1	Ser-Leu-Thr-Ser-Lys*-Ile-Asp-Ile-Thr-Leu	20 nmol
IP-2	Thr-Ser-Lys*-Ile-Asp-Ile-Thr-Leu	73 nmol

[†] Values are uncorrected for destruction or incomplete hydrolysis.

slight tendency to precipitate during the course of the reaction, and the enzyme isolated by gel filtration at the end of the experiment (265 min) was exclusively tetrameric. The specific activity of the enzyme was 180 units/mg, and the number of amino groups remaining unlabelled by methyl acetimidate was 1.10 mol/mol of enzyme subunit.

After carboxymethylation of all analytical samples and digestion for 4 h with chymotrypsin, a satisfactory two-dimensional separation of the resulting peptides was achieved (Fig. 4). The distribution of lysine-containing peptides from chloramphenicol acetyltransferase_{R387} is different from that obtained from the R429-specified variant (Fig. 2), even when the former enzyme is digested with chymotrypsin and trypsin. The double digestion alters the position of only two radioactive peptides. It is therefore unlikely that many of the lysine-containing sequences of chloramphenicol

Fig. 3. Amidination of native chloramphenicol acetyltransferase_{R387} with methyl acetimidate

Native chloramphenicol acetyltransferase_{R387} was amidinated with 50 mM-methyl acetimidate as described in the text. A second addition of fresh reagent (final concn. 50 mM) was made at the point denoted by the arrow. For key to symbols, see the legend to Fig. 1.

acetyltransferase_{R387} will be similar to those of chloramphenicol acetyltransferase_{R429}.

Although amidination of chloramphenicol acetyltransferase_{R387} appeared to leave two amino groups available for modification after denaturation, the incorporation of ¹⁴C was noted to be only 1.1 mol/mol of subunit. The anomaly was resolved by the isolation and characterization of these two peptides (IP-1 and IP-2) as described below. The demonstration that peptide IP-1 was a proteolytic precursor of IP-2 eliminated the possibility that the R387 variant of chloramphenicol acetyltransferase possesses more than one inaccessible amino group in each subunit.

Identification of the 'buried' lysine groups in chloramphenicol acetyltransferase_{R387}

Amidinated tetrameric chloramphenicol acetyltransferase (8 mg) was radioamidinated under denaturing conditions, desalted and *S*-carboxymethylated as described for the enzyme specified by plasmid R429. After extensive dialysis, the protein was digested with chymotrypsin for 4 h in 50 mM-NH₄HCO₃. The resulting clear solution was fractionated by gel filtration on a Sephadex G-25 column in the same buffer, and the single ¹⁴C-containing peak was pooled and freeze-dried. The constituent peptides were separated by descending chromatography in butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.) on Whatman 3MM paper. The two radioactive peptides were excised and electrophoresed on the same paper at pH 2.1.

Fig. 4. Radioautographs of the two-dimensional 'maps' of peptides generated by proteolysis of amidinated chloramphenicol acetyltransferase_{R387}

Samples of amidinated chloramphenicol acetyltransferase_{R387} were prepared for proteolysis in the manner described in the legend of Fig. 2, and digestion was carried out with chymotrypsin. The resulting peptides were analysed by electrophoresis and chromatography on silica gel. The average number of amino groups remaining unmodified per subunit in the native enzyme is shown at each time interval.

Peptide IP-2, detected by radioautography, was cut with care from one of two contiguous fluorescamine-positive bands; peptide IP-1 was similarly detected. Both peptides were analysed for amino acid composition and sequence (Table 1). Peptides IP-1 and IP-2 are clearly proteolytic variants of the same region of the primary structure.

These results therefore indicate that a single lysine residue in chloramphenicol acetyltransferase_{R387} is inaccessible to modification by methyl acetimidate in the native enzyme. As the complete primary structure of this enzyme variant is not yet known, it is not possible to assign a residue number to the lysine residue present in peptides IP-1 and IP-2, although a tentative estimate of its position may be made by comparison of the surrounding sequence with that of the primary structure of the type-I enzymes (see the Discussion section). It is clear that there are no obvious similarities of sequence between peptides IP-1 and IP-2. This is perhaps surprising in view of the close similarity of function between the two enzymes under investigation and the ease with which

they associate *in vitro* and *in vivo* to form a mixed tetramer with full catalytic activity (Shaw *et al.*, 1972).

Reaction of chloramphenicol acetyltransferase_{R429} with citraconic anhydride

The reaction of chloramphenicol acetyltransferase_{R429} with citraconic anhydride is extremely rapid under the conditions used. Catalytic activity is decreased to 0.2% of its original value within 30 s of incubation. Analysis of the modified protein product of the citraconylation reaction by gel electrophoresis under non-denaturing conditions (Fig. 5) demonstrates that incorporation of citraconyl groups is probably complete within 1 min of incubation. The electrophoretic mobility of the modified enzyme does not increase after this time. Prolonged incubation of the enzyme with citraconic anhydride did not effect any change in the mobility of the modified protein, and the sharpness of the tetramer band suggests homogeneity of substitution.

The gels in Fig. 5 also serve to demonstrate a

Fig. 5. Reaction of chloramphenicol acetyltransferase_{R429} with citraconic anhydride

Chloramphenicol acetyltransferase_{R429} was incubated with 55 mM-citraconic anhydride as described in the text. Samples of the reaction mixture were withdrawn at the times indicated and quenched with 5 vol. of 20 mM-Tris/HCl buffer, pH 7.8, containing 2-mercaptoethanol (0.1 mM). A second addition of anhydride was made after 160 min. The enzyme samples (30 μ g) were analysed by polyacrylamide-gel electrophoresis under non-denaturing conditions (gel composition: 12.5% T, 2.6% C). Migration is downward toward the anode. The R_m of the modified tetramer band (0.53) is constant after 1 min of modification, suggesting that complete substitution of the enzyme occurs within this time. Slower-migrating bands have apparent molecular weights which suggest that they are aggregates of the tetramer. The diffuse protein bands migrating near the dye front probably comprise denatured protein. The minor band migrating ahead of chloramphenicol acetyltransferase_{R429} tetramer is due to the accidental contamination of the preparation by the type-III chloramphenicol acetyltransferase variant specified by plasmid R387. The latter also remains tetrameric on modification (results not shown). The affinity resin used to purify the R429-specified variant had been used in previous experiments for the purification of chloramphenicol acetyltransferase_{R387}. Although the resin was subsequently washed with solutions containing chloramphenicol and 1 M-NaCl, it is apparent that small amounts of the R387 protein remained bound and were eluted subsequently.

phenomenon which appears to be exclusive to type-I variants (Fitton *et al.*, 1978) of unmodified chloramphenicol acetyltransferase. The slower-migrating bands observed on the first gel are likely to be

aggregates of the native tetramer, and they cannot be detected by gel filtration. The oxidizing conditions of the discontinuous gel system induce aggregation, especially at higher loadings of protein, but dissociation to the native tetrameric state can be induced by treatment with reducing agent before the second electrophoresis in a two-dimensional gel system (results not shown). Type-III variants of the enzyme neither aggregate in the gel system, nor do they form mixed aggregates with type-I enzymes (Packman, 1978).

The relevance of the above observations to this experiment is that citraconylation of chloramphenicol acetyltransferase_{R429} does not inhibit the aggregation, although the repulsive forces between modified tetramers must be substantially increased over that expected for the native enzyme. The concentration of the protein during the stacking procedure should be sufficiently high to overcome these forces and must allow extensive contacts between tetramers. It is somewhat surprising, therefore, that native and citraconylated enzyme do not form mixed aggregates when mixed and loaded on to the same gel (Fig. 6).

Characterization of citraconyl-chloramphenicol acetyltransferase_{R429}

Gel filtration of citraconylated chloramphenicol acetyltransferase_{R429} on Sephadex G-75 shows that the modified protein is predominantly (85%) in the tetrameric form. The remaining 15% of the loaded protein does not appear as a discrete monomer peak at the V_t of the column. Electrophoresis of the pooled excluded material under non-denaturing conditions produces gels that are virtually identical with those shown in Fig. 5. It seems likely, therefore, that the protein appearing on these gels as diffuse bands migrating close to the dye front comprises a heterogeneous population of partially denatured enzyme. Such species appear to be distributed throughout the gel-filtration profile and are therefore collected to some extent in the pooled excluded material.

The isolated citraconyl-chloramphenicol acetyltransferase_{R429} is therefore 'contaminated' with a small percentage of denatured, soluble protein. We estimate that this should introduce an error of not more than 5% in the estimation of free lysine content of the protein by radioamidation.

Amidination of citraconylated enzyme with methyl [1-¹⁴C]acetimidate (sp. radioactivity 0.08 Ci/mol) under denaturing conditions shows an incorporation of ¹⁴C of 1.15 mol/mol of enzyme subunit. A single amino group per subunit therefore remains 'buried' by virtue of sequestration within the tertiary or quaternary structure of the modified enzyme and is thus unavailable for modification by citraconic anhydride. The identification of this amino group by

Fig. 6. Removal of citraconyl groups from citraconyl-chloramphenicol acetyltransferase_{R429}. Citraconyl-chloramphenicol acetyltransferase_{R429} was incubated at pH 6 and 37°C for 3 days. Regeneration of free amino groups (●) was assayed as described in the text. The removal of citraconyl groups was accompanied (Fig. 6c) by a regain of enzyme activity (○) and a decrease in negative charge of the protein, as shown by native-gel electrophoresis (12.5% T, 2.6% C). Precipitation was visually evident after 2 h, concomitant with the disappearance on the gels of fast-migrating heterogeneous material running at or near the dye front. After 72 h incubation at pH 6, the regenerated enzyme does not have the electrophoretic mobility of the native enzyme, but has regained 94% of the catalytic activity of untreated enzyme and 96% of the free amino group content. Gel I represents a sample of unmodified native enzyme; gel II represents a mixture of unmodified and fully citraconylated enzyme. No mixed aggregates ('hybrids') of the two forms of enzyme are apparent. Gels 2–5 (from left to right) were loaded with 15–20 µg of protein, and the remaining gels with 30 µg.

proteolytic digestion and peptide 'mapping' reveals that it resides in peptide IR-1 (Fig. 7; 'map' 1) and is the same residue that is unavailable for modification by methyl acetimidate in the native enzyme, namely lysine-136 (Packman & Shaw, 1981). Two independent methods have therefore produced consistent results, reinforcing the validity of both

methods in the examination of lysine topography in enzymes.

Removal of citraconyl groups from modified chloramphenicol acetyltransferase_{R429}

The observed contrast between the ease of inactivation of chloramphenicol acetyltransferase by

Fig. 7. Radioautographs of two-dimensional 'maps' of peptides generated by proteolysis of amidinated chloramphenicol acetyltransferase_{R429}

Citraconylated chloramphenicol acetyltransferase_{R429} was deblocked by incubation at pH 6. Samples of protein were withdrawn from the reaction mixture at the times shown and free amino groups were amidinated with methyl [1-¹⁴C]acetimidate under denaturing conditions. After removal of remaining citraconyl groups by dialysis against 5% (v/v) acetic acid, the protein was modified with methyl acetimidate as described in the text. The samples were *S*-carboxymethylated, digested with chymotrypsin (1%, w/w) and trypsin (1%, w/w) for 2 h each in 50 mM-NH₄HCO₃ at 37°C. The resulting peptides were analysed by electrophoresis at pH 6.5 and chromatography in butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.) on silica gel. The small circled areas near the centre of 'map' 1 represent the positions of the fluorescent markers [dansyl derivatives of arginine (neutral) and arginyl-arginine (adjacent to peptide IR-1)], which were mixed with the peptide digest before application. 'Map' 1, derived from fully citraconylated enzyme, shows the single unmodified lysine residue to be located in peptide IR-1. This peptide contains lysine-136 (Packman & Shaw, 1981). 'Maps' 2–6, bearing a heavier peptide loading than 'map' 1, show the rate of hydrolysis of citraconyl-amide bonds to be non-uniform with respect to individual amino groups. Minor differences exist between the 'maps' derived from regenerated ('map' 6) and control ('map' 7) enzyme; these are denoted by arrows, and may represent peptides containing non-lysine modifications. The estimation of free amino-group content of the sample represented by 'map' 3 is probably in error. 'Maps' 2 and 4, and Fig. 6, clearly show this sample to be more deblocked than the sample taken at 1 h. The radioactive peptide IR-1 was shown to be identical with its counterpart derived from the amidination experiments described in the legend to Fig. 2 on the basis of thin-layer co-chromatography and co-electrophoresis. Peptide IR-1 has the sequence Phe-Pro-Lys-Gly-Phe.

citraconic anhydride and its resistance to dissociation after citraconylation prompted a study of the removal of citraconyl groups from the fully modified protein and the properties of the re-activated enzyme.

Citraconylated enzyme was incubated at 37°C and pH 6 for 3 days, over which time regain of catalytic activity reached a maximum, and regeneration of free amino groups attained a value equivalent to 96% of that of the untreated enzyme (Fig. 6). A control sample of enzyme incubated under the same conditions showed a 10% loss of catalytic activity over the 3-day period, during which time

there was no gross evidence of microbial contamination.

The reappearance of catalytic activity did not parallel the regeneration of free amino groups, the latter being the more rapid and virtually complete in less than 6 h. A possible explanation of this observation would invoke (a) an essential role for one or a few lysine residues which are required either directly or indirectly for full catalytic activity and (b) the slow removal of citraconyl groups from such *N*⁶-amino groups, perhaps owing to ionic interactions of citraconyl-lysine residues with contiguous amino acids such as arginine or histidine residues. A

detailed kinetic analysis of this hypothesis cannot, however, be made with the small number of data points available for the regeneration of free amino groups.

Monitoring the regeneration of amino groups by proteolysis and peptide 'mapping' of radiolabelled protein provides an independent assessment of the rate of hydrolysis of individual citraconyl amide bonds (Fig. 7). The rate of intramolecular catalysis, proposed to facilitate the removal of citraconyl and maleyl groups (Butler *et al.*, 1969), is undoubtedly being influenced by neighbouring amino acids.

The results of native-gel electrophoresis of samples of chloramphenicol acetyltransferase_{R429} at various stages of regeneration (Fig. 6) agree with the radioamidation and peptide-'mapping' data, demonstrating an initially rapid regain of free lysine groups (i.e. a rapid decrease in net electronegativity of the protein) followed by a slower phase. The loss of rapidly migrating heterogeneous protein coincides with the appearance of precipitate in the reaction mixture.

At the point of attainment of maximum catalytic activity, the regenerated protein present in the reaction mixture supernatant (45% yield) remains tetrameric. This protein appears electrophoretically heterogeneous and is slightly more electronegative than untreated enzyme, but it possesses a specific activity of 183 units/mg of protein. This is equivalent to 94% of the specific activity of the control enzyme (195 units/mg). Comparison of the peptide 'maps' of native and regenerated chloramphenicol acetyltransferase_{R429} (Fig. 7, 'maps' 7 and 6 respectively) shows that there are a few minor differences between the lysine-containing peptides in each enzyme. Two low-yield peptides (indicated by arrows) are present in the regenerated enzyme but are absent from the native enzyme. A third difference may exist in the neutral peptide fraction. Each could represent peptides with modified hydroxyamino acids or cysteine residues which were not unblocked by treatment with 5% acetic acid. Modified thiol groups are unlikely, however, since there was no change in electrophoretic mobility after treatment with neutral hydroxylamine (results not shown).

Discussion

The utility of methyl acetimidate as a probe of the topographic distribution of lysine groups on the 'surface' of glyceraldehyde 3-phosphate dehydrogenase was clearly demonstrated by Lambert & Perham (1977). When the chemical-modification results were compared with X-ray-crystallographic data, it was concluded that the lysine residue inaccessible in the native enzyme to modification by methyl acetimidate was located at the subunit-subunit interface, where it participated in ion-pair

formation with an aspartic acid residue. Lysine residues on the 'surface' of the enzyme showed differential reactivity towards methyl acetimidate. Some of these residues were involved in ionic interactions with charged acidic groups; others were judged to be partially 'buried' in hydrophobic pockets.

One may draw similar conclusions about the interactions of lysine residues in chloramphenicol acetyltransferase. The differential reactivity of 'surface' lysine residues towards methyl acetimidate reflects their probable ionic interaction with neighbouring anionic groups and the extent of their interaction with solvent. It is tempting to speculate that the lysine residue that cannot be modified by imidoester in each of the native enzymes is located at a subunit interface. Indeed, towards examining this possibility, attempts were made to determine whether any lysine residues were still unavailable for modification in resolved subunits. The strategy was to dissociate chloramphenicol acetyltransferase reversibly into its subunits by citraconylation (Dixon & Perham, 1968; Gibbons & Perham, 1970), thus exposing the subunit interfaces. One may then observe whether any lysine residues are buried within the interior of the monomers by radioamidation under denaturing conditions. Surprisingly, the quaternary structure of chloramphenicol acetyltransferase_{R429} remains intact upon complete citraconylation, and lysine-136 is still unreactive. In the absence of direct evidence, therefore, one can only infer the possibility of the buried lysine residue being located at a subunit interface.

An effort has been made to establish the atomic co-ordinates of the type-I chloramphenicol acetyltransferase specified by plasmid JR66b (Liddell *et al.*, 1978). When the results are available, the interactions of lysine-136 with other residues should become clear. The crystallographic data for chloramphenicol acetyltransferase_{R387} (I. Liddell & I. D. A. Swan, unpublished work) are not as advanced as those for the type-I enzyme, but the determination of the three-dimensional structure of these two enzyme variants will be a major advance in the understanding of the diversity in structure which is becoming increasingly apparent between the types-I and -III variants of chloramphenicol acetyltransferase (Fitton *et al.*, 1978; Zaidenzaig *et al.*, 1979).

Such differences, reflected in the experiments reported here by the dissimilarities between the thin-layer 'maps' of lysine-containing peptides from the two enzymes, are evident in the sequence information available to date on the type-III enzyme (N. M. C. Kaye, L. C. Packman & W. V. Shaw, unpublished work). Although regions of homology exist between the two variants, there are several

sequences within the structure of chloramphenicol acetyltransferase_{R387} that cannot presently be aligned with that of the type-I variant. The alignment of peptide IP-2 from the type-III enzyme with the sequence of the type I enzyme is therefore provisional:

Type-I enzyme	-Asn-Gln-Thr-Val-Gln-Leu-Asp-Ile-Thr-Ala-
Type-III enzyme	-Ser-Leu-Thr-Ser-Lys-Ile-Asp-Ile-Thr-Leu-
(peptide IP-2)	

The *N*-terminal-sequence data for the types-I and -III enzymes (Fitton *et al.*, 1978) show that the first residue of type-III chloramphenicol acetyltransferase aligns with residue 6 of the type-I (prototype) enzyme. The provisional assignment of the lysine residue in peptide IP-2 may therefore be made with some confidence as lysine-38. The buried lysine residue in chloramphenicol acetyltransferase_{R429} is lysine-136.

If this alignment of peptide IP-2 with the prototype sequence is valid, and if the primary structure of the type-III enzyme aligns with that of the type-I enzyme over a large percentage of its length, then any similarity of function of the buried lysine residues in the two types of enzyme may be by virtue of polypeptide-chain folding in such a way as to place residues 136 (type I) and 38 (type III) in similar environments relative to the tertiary structure of the enzyme. Should this be the case, one would predict that both lysine residues would be inaccessible to modification by methyl acetimidate in a native hybrid tetramer composed of two subunits of each enzyme variant. Experiments designed to test this prediction have yielded results which do not fully support such a view (Packman & Shaw, 1981).

The main conclusions to be drawn from the citraconylation experiments seem clear. Exhaustive modification of the type-I chloramphenicol acetyltransferase yields a protein which is still tetrameric but which is lacking in catalytic activity (0.02% of control) and in which lysine-136 remains unmodified.

Removal of citraconyl groups from the enzyme results in an electrophoretically heterogeneous protein which, within experimental error, possesses full catalytic activity. Non-lysine substitution with citraconic anhydride appears to be have occurred, but with no adverse effect on the regain of catalytic activity. Such side reactions appear to have occurred within the first minute of modification.

Comments on the quaternary structure of chloramphenicol acetyltransferase

Although resistance of chloramphenicol acetyltransferase_{R429} to dissociation by citraconic anhydride was disappointing, the observation has provoked subsidiary questions which may be of more

general importance. This result mirrors the stability of the enzyme to variations in temperature (a 60°C heat step is often used in the enzyme-purification procedure) ionic strength (10 mM–1 M-NaCl) and pH over the range 6–9 (Shaw, 1975; Zaidenzaig & Shaw, 1976; Packman, 1978). Under all these

conditions, chloramphenicol acetyltransferase has a stable quaternary structure. By contrast, although the enzyme is unfolded by high concentrations of urea and guanidinium chloride, renaturation of the protein upon slow dilution of the denaturing agent is poor and recovery of catalytic activity is rarely more than 20% for the type-I enzyme, and less (3%) for the type-III variant (W. V. Shaw & L. C. Packman, unpublished work).

The above observations are consistent with the proposal that the stabilization of the quaternary structure of chloramphenicol acetyltransferase involves very strong binding forces between the subunits which cannot easily be disrupted by perturbing the net surface charge of the enzyme via changes in ionic strength, pH, or by chemical modification. It seems likely that particularly strong hydrophobic interactions exist in the chloramphenicol acetyltransferase system, and work must now be directed at such non-ionic interactions; the results should lead to hypotheses which may ultimately be tested by X-ray diffraction.

Note Added in Proof (Received 3 December 1980)

Recent work has shown that the lysine residue of peptide IP-2 is indeed located at position 38 in the covalent sequence of chloramphenicol acetyltransferase_{R387} (N. M. C. Kaye, J. W. Keyte & W. V. Shaw, unpublished work).

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