The use of naturally occurring hybrid variants of chloramphenicol acetyltransferase to investigate subunit contacts

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1. Hybrids of the tetrameric enzyme chloramphenicol acetyltransferase (EC 2.3.1.28) were formed *in vivo* in a strain of *Escherichia coli* which harbours two different plasmids, each of which normally confers chloramphenicol resistance and specifies an easily distinguished enzyme variant (type I or type III) which is composed of identical subunits. Cell-free extracts of the dual-plasmid strain were found to contain five species of active enzyme, two of which were the homomeric enzymes corresponding to the naturally occurring tetramers of the type-I (β_4) and type-III (α_4) enzymes. The other three variants were judged to be the heteromeric hybrid variants ($\alpha_3\beta$, $\alpha_2\beta_2$, $\alpha\beta_3$). 2. The $\alpha_3\beta$ and $\alpha_2\beta_2$ hybrids of chloramphenicol acetyltransferase were purified to homogeneity by combining the techniques of affinity and ion-exchange chromatography. The $\alpha\beta_3$ variant was not recovered and may be unstable *in vitro*. 3. The unique lysine residues that could not be modified with methyl acetimidate in each of the native homomeric enzymes were also investigated in the heteromeric tetramers. 4. Lysine-136 remains buried in each β subunit of the parental (type I) enzyme and in each of the hybrid tetramers. Lysine-38 of each α subunit is similarly unreactive in the native type-III chloramphenicol acetyltransferase (α_4), but in the $\alpha_2\beta_2$ hybrid lysine-38 of each α subunit is fully exposed to solvent. Another lysine residue, fully reactive in the α_4 enzyme, was observed to be inaccessible to modification in the symmetrical hybrid. The results obtained for the $\alpha_3\beta$ enzyme suggest that lysine-38 in two subunits and a different lysine group (that identified in the $\alpha_2\beta_2$ enzyme) in the third α subunit are buried. 5. A tentative model for the subunit interactions of chloramphenicol acetyltransferase is proposed on the basis of the results described.

Methyl acetimidate can be a useful reagent in enzyme-modification studies which aim to determine the chemical reactivity and function of amino groups in a native protein (Lambert & Perham, 1977; Lambert *et al.*, 1977; Packman & Shaw, 1981). The interpretation of the observed reactivity in terms of physical interactions in any given case is, however, often limited by the absence of complementary data from X-ray-diffraction or other studies.

Apart from this limitation, identification of a unique lysine residue that is totally unavailable for modification in a native enzyme strongly suggests that the residue in question is located in an

environment that is shielded from the solvent. Whether such a site lies within the interior of a subunit or at an interface between subunits is often difficult to determine unambiguously by chemical methods. In principle, this could be investigated by reversibly dissociating an enzyme into its constituent subunits under conditions wherein the native tertiary structure of monomers was preserved, thus exposing only the interface regions to solvent.

Attempts to achieve this objective with chloramphenicol acetyltransferase (EC 2.3.1.28) by modification with citraconic anhydride (Dixon & Perham, 1968) were unsuccessful (Packman & Shaw, 1981). Two variants of the enzyme specified by plasmids R429 (type I) and R387 (type III) each have a single lysine residue per subunit in the native enzyme which is unreactive towards methyl acetimidate (Packman & Shaw, 1981). The residue (lysine-136) of the type-I variant (chloramphenicol acetyl-

Abbreviation used: SDS, sodium dodecyl sulphate.

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transferase_{R429}) that cannot be amidinated is also inaccessible to citraconic anhydride (Packman & Shaw, 1981). The residue (lysine-38) of the type-III variant (chloramphenicol acetyltransferase_{R387}) that cannot be amidinated has not been investigated by acylation.

Although the primary structures of the type-I and type-III variants possess several regions of homology, the 'buried' lysine residues are clearly in different positions in the linear sequence, raising the question of their functional homology (Packman & Shaw, 1981). The use of a hybrid enzyme, comprising two subunits of chloramphenicol acetyltransferase_{R429} and two of chloramphenicol acetyltransferase_{R387}, has been described previously and could be a useful approach to this problem (Shaw *et al.*, 1972).

The present paper describes the isolation and purification of hybrid enzymes, formed *in vivo* from a dual-plasmid strain of *Escherichia coli*, by affinity and ion-exchange chromatography. The results indicate that three hybrid forms ($\alpha_3\beta$, $\alpha_2\beta_2$, $\alpha\beta_3$) of chloramphenicol acetyltransferase are present in the cell as well as the homomeric forms, chloramphenicol acetyltransferase_{R429} (β_4) and the R387-specified variant (α_4).

Amidination studies performed on the $\alpha_2\beta_2$ and $\alpha_3\beta$ hybrid enzymes indicate that heteromeric and homomeric association of subunits involve different interactions.

Materials and methods

Chemicals

Methyl acetimidate was synthesized as described by Hunter & Ludwig (1962); methyl [1-¹⁴C]-acetimidate was prepared by the method of Bates *et al.* (1975), and had a specific radioactivity of 0.08 Ci/mol. Ampicillin was obtained from Beechams Research Laboratories, Brentford, Middx., U.K.; streptomycin sulphate, chloramphenicol and triethanolamine/HCl were purchased from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. DEAE-cellulose (DE52) was purchased from Whatman Biochemicals, Maidstone, Kent, U.K. All other chemicals were of analytical grade.

Bacterial strains

The strain of *Escherichia coli* used in this work was a dual-plasmid isolate, which has been described previously (Shaw *et al.*, 1972). *E. coli* strain J53 (R429; R387) contains two plasmids. Plasmid R429 codes for resistance to ampicillin, tetracycline and chloramphenicol, and R387 codes for resistance to chloramphenicol and streptomycin (Hedges, 1975).

Assay of chloramphenicol acetyltransferase

Chloramphenicol acetyltransferase activity was measured at 37°C by the spectrophotometric method (Shaw, 1975). One enzyme unit is defined as that amount of enzyme which catalyses the formation of 1 μ mol of product/min at 37°C.

Preparation of chloramphenicol acetyltransferase variants from *E. coli* J53 (R429; R387)

(a) *Preparation of crude extract.* The dual-plasmid *E. coli* strain was cultured at 37°C in twelve shaker flasks (nominal size 2 litres) containing a total of 15 litres of Oxoid nutrient broth (double strength) supplemented with glycerol (0.3%, v/v). To select against single-plasmid segregants of the organism, the inoculum was grown in the same broth containing chloramphenicol (50 μ g/ml), ampicillin (25 μ g/ml) and streptomycin sulphate (25 μ g/ml). Cells were harvested in the early stationary phase of growth by passage through a Sharples continuous-flow centrifuge and washed with distilled water.

The packed cell paste (75.4 g wet wt.) was resuspended, with a blender, in 200 ml of 50 mM-Tris/HCl buffer, pH 7.8, containing 2-mercaptoethanol (0.1 mM), 0.2 mM-chloramphenicol, phenylmethanesulphonyl fluoride (50 μ g/ml) and deoxyribonuclease I (1 μ g/ml). Cells were broken by extrusion through a small orifice at 83 MPa (12 000 lb/in²) by using an Aminco pressure cell cooled to 0°C, and the resulting non-viscous extract was centrifuged at 17 000 g for 10 min to remove cell debris. The pellet was resuspended in a small volume of the same buffer and subjected to a second cycle of breakage. Supernatants were pooled and heated with stirring to 60°C, that temperature being maintained for 5 min. The extract was cooled, with stirring, to 3°C and centrifuged at 50 000 g for 30 min to yield the crude extract.

Samples of the extract were analysed by polyacrylamide-gel electrophoresis under non-denaturing conditions. The gels were stained for chloramphenicol acetyltransferase activity by using the phenazine/tetrazolium method as described below. Five species of chloramphenicol acetyltransferase were observed: two parental forms corresponding to the type-III (R387) and type-I (R429) variants (termed α_4 and β_4) and three hybrid forms (termed $\alpha_3\beta$, $\alpha_2\beta_2$, $\alpha\beta_3$).

(b) *Affinity chromatography of chloramphenicol acetyltransferase in the crude extract of *E. coli* J53 (R429; R387).* The affinity resin, comprising chloramphenicol base coupled to aminohexanoyl-Sepharose-4B, was prepared as described by Zaidenzig & Shaw (1976). The resin possessed the following substitutions per ml of settled gel: 4.7 μ mol of carboxy groups and 5.9 μ mol of chloramphenicol base in amide linkage (Packman & Shaw, 1981).

The crude extract was passed at 4°C through a

column containing 50 ml of affinity resin. The column was washed with 50 mM-Tris/HCl buffer, pH 7.8, containing 2-mercaptoethanol (0.1 mM). The same buffer was used in all succeeding steps, with additions of solute as necessary. A total of 87% of the applied chloramphenicol acetyltransferase activity bound to the resin. The unbound activity, which was discarded, was predominantly the type-III (α_4) parental variant, specified by plasmid R387, which is known to have a lower affinity for the bound ligand (Zaidenzaig & Shaw, 1976).

When the $A_{280}^{1\text{cm}}$ of the effluent buffer fell to less than 0.07 (8 column volumes of buffer) elution of some of the bound chloramphenicol acetyltransferase from the resin was effected by the application of a linear gradient of chloramphenicol (0–10 mM) in buffer (400 ml). At the end of the gradient, the column was washed with buffer alone to remove chloramphenicol. All fractions were assayed for enzyme activity and analysed by polyacrylamide-gel electrophoresis under native conditions, with staining for protein. Fractions enriched in each of the α_4 , $\alpha_3\beta$ and $\alpha_2\beta_2$ species of chloramphenicol acetyltransferase were identified, pooled and stored at 4°C. These pools were termed DE1, DE2 and DE3 respectively (see the Results and discussion section). Contamination of the fractions by other acidic proteins was less than 5% as judged by electrophoresis under native conditions.

Further elution steps were required to complete the elution of the $\alpha_2\beta_2$, $\alpha\beta_3$ and β_4 species of chloramphenicol acetyltransferase from the resin. After washing the resin in the low-salt buffer as described above, elution of the residual activity was achieved by stepwise increases in ionic strength in the presence of chloramphenicol. The column was washed with buffer containing 50 mM-NaCl (until the effluent $A_{280}^{1\text{cm}}$ was less than 0.10; 15 column values) and then with the gradient of chloramphenicol in the same NaCl-containing buffer (350 ml). The $\alpha_2\beta_2$ and β_4 variants of chloramphenicol acetyltransferase were clearly identified by electrophoresis in the collected fractions, but only trace amounts of $\alpha\beta_3$ enzyme were observed. Fractions enriched in $\alpha_2\beta_2$ enzyme were pooled and termed pool DE4. The column was washed with buffer alone.

A final elution step, in the above manner, with 75 mM-NaCl, achieved elution of the β_4 species corresponding to chloramphenicol acetyltransferase_{R429}. The $\alpha\beta_3$ variant constituted 1–2% of the total enzyme activity eluted, a yield judged to be too low to justify isolation of the enzyme for the studies to be described.

(c) *Ion-exchange chromatography of parental and hybrid chloramphenicol acetyltransferase variants.* Ion-exchange chromatography on DEAE-cellulose (DE52) was necessary to achieve complete separation of the variants of chloramphenicol

acetyltransferase and for the removal of contaminating basic and acidic proteins.

Optimal resolution was obtained by independently chromatographing pools DE1–DE4. Pools DE1, DE2 and DE3 were loaded directly on to a column (bed dimensions 20 cm × 2.5 cm diameter) equilibrated with 50 mM-Tris/HCl buffer, pH 7.8, containing 2-mercaptoethanol (0.1 mM) and 0.2 mM-chloramphenicol, at 4°C. The inclusion of chloramphenicol was necessary for good recovery of enzyme activity. Pool DE4 was diluted with an equal volume of equilibrating buffer before loading, to lower the NaCl concentration in this sample to 25 mM.

For each separation the column was first washed with equilibrating buffer before elution of chloramphenicol acetyltransferase with a linear gradient (1500 ml) of 0.1–0.3 M-NaCl in the same buffer. All fractions were analysed by enzyme assay and electrophoresis under non-denaturing conditions. Hybrid enzymes were observed to have elution properties intermediate between those of the two parental variants.

Fractions from each DEAE-cellulose experiment that were found to contain a single species of enzyme were pooled, yielding samples of fully resolved α_4 , $\alpha_3\beta$, $\alpha_2\beta_2$ and β_4 variants of chloramphenicol acetyltransferase. Each variant was shown to be homogeneous by polyacrylamide-gel electrophoresis under non-denaturing and denaturing conditions.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis in the presence of 0.1% (w/v) SDS was carried out in the buffer system of Laemmli (1970). Electrophoresis under non-denaturing conditions was carried out in the same buffer system but with the omission of detergent. In this instance, sample buffer was replaced by 50 mM-Tris/HCl buffer, pH 7.8, containing 2-mercaptoethanol (0.1 mM). Gel compositions are described by the nomenclature of Hjerten (1962).

Non-denaturing gels were stained for 30 min with 0.123% (w/v) Naphthalene Black in 0.1 M-acetic acid and destained by diffusion in 5% (v/v) acetic acid. SDS-containing gels were stained overnight with 0.0125% (w/v) Coomassie Brilliant Blue R dissolved in 40% (v/v) methanol and 70% (v/v) acetic acid in water. Destaining was by diffusion in 7% (v/v) acetic acid.

Identification of chloramphenicol acetyltransferase in electrophoretograms

Detection of chloramphenicol acetyltransferase activity in non-denaturing gel electrophoresis of crude extracts was by the method of Shaw & Brodsky (1968). This assay utilizes the coupled reduction of Nitro Blue Tetrazolium to the insoluble

formazan by free CoA, via an intermediate electron carrier, phenazine methosulphate. Gels incubated with the dyes and acetyl-CoA in the absence of chloramphenicol were used as controls.

Protein determination

Protein in crude extracts was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Homogeneous preparations of enzyme were estimated by amino acid analysis by using the known amino acid composition of the enzymes concerned (Packman, 1978).

Amino acid analysis

Hydrolysis of protein was carried out in the presence of 6M-HCl, containing 10 μ M-phenol, *in vacuo* at 105°C for 24 h. Amino acids were quantified by using a single-column analyser (Locarte) operating with sodium citrate buffers.

Amidination of chloramphenicol acetyltransferase

Samples of native parental and hybrid enzyme (approx. 1 mg/ml in 0.2M-triethanolamine/HCl, pH 8.5) were amidinated with 50 mM-methyl acetimidate at room temperature as described previously (Packman & Shaw, 1981). Modification was monitored by loss of enzyme activity only. The activities of the $\alpha_2\beta_2$ and β_4 enzymes were decreased to 40% of the initial value, and those of the α_4 and $\alpha_3\beta$ enzymes to 30% and 35% respectively.

A second addition of fresh reagent (50 mM final concn.) was made after 128 min, after which incubation was continued for a further 120 min. Excess reagent was removed by gel filtration on Sephadex G-75 equilibrated with the same buffer. Recovery of tetrameric enzyme was 90–100% for each enzymic species except β_4 , for which it was 80%.

Modified enzyme was amidinated by 0.1M-methyl [$1\text{-}^{14}\text{C}$]acetimidate under denaturing conditions in 0.2M-triethanolamine/HCl buffer, pH 8.5, containing 8M-guanidinium chloride. A second addition of fresh reagent was made after 2 h and modification continued for a further 2 h. The number of amino groups thus modified with radioactive reagent was estimated as described previously (Packman & Shaw, 1981).

Thin-layer electrophoresis and chromatography

Samples of salt-free amidinated protein were digested with 2% (w/w) chymotrypsin and 2% (w/w) trypsin for 2 h each in 50 mM- NH_4HCO_3 , pH 7.8, at 37°C. The resulting peptides were analysed by electrophoresis on silica gel at pH 6.5 and chromatography in butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.) at right angles to the direction of electrophoresis (Lambert & Perham, 1977; Packman & Shaw, 1981). Radio-labelled peptides were detected by radioautography.

Results and discussion

The first experiments on the hybridization *in vivo* of types-I and -III variants of chloramphenicol acetyltransferase revealed the predominance of a single symmetrical hybrid enzyme comprising two subunits of chloramphenicol acetyltransferase_{R429} (type I) and two of the R387-specified variant (type III). The same $\alpha_2\beta_2$ hybrid was observed when chloramphenicol acetyltransferase_{R429} (β_4) and chloramphenicol acetyltransferase_{R387} (α_4) were renatured together from denaturing concentrations of guanidinium chloride (Shaw *et al.*, 1972). The expected asymmetric heteromers were not detected.

In order to isolate sufficient quantities of hybrid enzyme to study the interaction of subunits by techniques of small-scale protein chemistry, an improved purification method was required. The approach used was first to reinvestigate and identify the number of chloramphenicol acetyltransferase variants present in the dual-plasmid strain of *E. coli*, containing active genes for both the R429- and R387-specified variants of the enzyme. Thereafter, the strategy was to separate the variants from one another and from other proteins by affinity chromatography and, if required, by a final ion-exchange-chromatography step. It was appreciated that success rested on exploiting fully the differential elution properties of each variant from the affinity resin.

Preparation of a crude extract of *E. coli* J53 (R429; R387)

During the early stages of purification, recovery of enzyme activity was less than expected. Purification of chloramphenicol acetyltransferase from *E. coli* J53 containing plasmid R429 or plasmid R387 alone has given reproducibly typical recoveries of chloramphenicol acetyltransferase after the dialysis step of 95–100% (Packman, 1978). From *E. coli* J53 (R429, R387), however, only 73% of the enzyme activity is recovered under the same conditions (Table 1). It is not clear whether the loss of activity from the dual-plasmid strain is due to properties of the extract such as enhanced proteinase activity or the innate instability of one or more of the hybrid forms of chloramphenicol acetyltransferase. The results described below suggest that the latter may prove to be a likely explanation.

Analysis of the crude extract by gel electrophoresis under non-denaturing conditions and localization of chloramphenicol acetyltransferase activity by the dye-reduction stain produced results which, in view of earlier studies, were unexpected. Five distinct bands were observed (Fig. 1). The mobilities of the slowest- and fastest-migrating bands corresponded to those of chloramphenicol acetyltransferase_{R429} (β_4) and chloramphenicol acetyltransferase_{R387} (α_4) respectively. Bands of intermediate mobility were

Table 1. Preparation of cell-free extract of *E. coli* J53 (R429; R387)

Preparation step	Volume (ml)	Chloramphenicol acetyltransferase activity (units/ml)	Total activity (units)	Protein (mg/ml)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification factor
Crude extract	190	213	40 280	59.3	11 250	3.6	100	1.0
Heat step	340	106	36 108	19.4	6600	5.5	90	1.5
Dialysis against 50 mM-Tris/HCl buffer, pH 7.8, containing 2-mercapto-ethanol (0.1 mM)	375	79	29 520	16.4	6150	4.8	73	1.3

Fig. 1. Non-denaturing gel electrophoresis of crude extract prepared from *E. coli* J53 (R429; R387)

Samples of crude extract (50 μ l) were electrophoresed in polyacrylamide gels (8.5% T, 2.6% C) under non-denaturing conditions. Reference gels of the same composition were run simultaneously bearing samples of chloramphenicol acetyltransferase_{R429} and chloramphenicol acetyltransferase_{R387} (5 μ g each). A reference (1) and a test (2) gel were stained for chloramphenicol acetyltransferase activity with the phenazine/tetrazolium stain. Five enzyme activities were identified in the crude extract, two of which co-chromatographed with the reference enzymes. The three enzymes of intermediate mobility were presumed to be hybrids, as labelled. To monitor for general deacylase and reducing activities in the extract, a parallel set of gels was stained with histochemical stain lacking chloramphenicol. The results confirmed the bands identified in gel 2 as being chloramphenicol acetyltransferase-specific.

presumed to be hybrids of subunit composition $\alpha\beta_3$, $\alpha_2\beta_2$ and $\alpha_3\beta$, with decreasing net negative charge. Shaw *et al.* (1972) observed only the $\alpha_2\beta_2$ variant. The resolution of the hybrid forms possibly depends on the electrophoretic system employed.

Affinity chromatography of the crude extract of E. coli J53 (R529; R387)

Passage of the crude extract through affinity resin resulted in a 90% retention of chloramphenicol acetyltransferase activity. Unbound enzyme found in the break-through fraction was predominantly chloramphenicol acetyltransferase_{R387}, and its partial removal at this stage allowed a more efficient separation of $\alpha_3\beta$ enzyme from the α_4 variant upon subsequent ion-exchange chromatography.

Elution of chloramphenicol acetyltransferase from the affinity resin was a three-stage process requiring increasing salt concentrations in the elution buffer (see the Materials and methods section). Recovery of the hybrid enzyme was essentially complete after stage 2 (Fig. 2).

An important feature of the elution profiles is that each enzyme variant chromatographs differently, suggesting that the interaction of chloramphenicol acetyltransferase variants with the resin is not simply a function of the interactions of their active sites with the bound ligand. The α_4 and $\alpha_3\beta$ variants are completely recovered by elution with 50 mM buffer and chloramphenicol (0–10 mM). The $\alpha_2\beta_2$ variant requires the presence of 50 mM-NaCl for maximum recovery, and the β_4 variant 75 mM-NaCl. Overall recovery of the applied activity was 78% (51% overall) (Table 2). The $\alpha\beta_3$ heteromer behaves chromatographically like the β_4 enzyme, but the low recovery of this hybrid compared with the latter variant casts some doubt on its stability as a native tetramer *in vitro* and, by inference, *in vivo*.

The electrophoretic mobility of each purified variant was seen to be in good agreement with the results of non-denaturing gel electrophoresis of the crude extract, wherein staining for enzyme activity was used to identify each variant.

Ion-exchange chromatography of chloramphenicol acetyltransferase variants

Resolution of chloramphenicol acetyltransferase variants was achieved by anion-exchange chromatography (Fig. 3). The recovery of bound enzyme was 60–70% for each chromatographic separation. Pooling the relevant fractions from each experiment

Fig. 2. Affinity chromatography of crude extract from *E. coli* J53 (R429; R387)

A crude extract of *E. coli* J53 (R429; R387) was prepared as described in the Materials and methods section and subjected to affinity chromatography at 4°C, with 50 ml of resin (see Table 2). Extract (375 ml) was applied and the column was washed with 400 ml of 50 mM-Tris/HCl buffer, pH 7.8, containing 2-mercaptoethanol (0.1 mM). (a) Eluent: linear gradient (400 ml) of 0–10 mM-chloramphenicol in the above buffer. The flow rate was 5 ml/min. Each fraction (5 ml) was analysed by non-denaturing gel electrophoresis (8.5% T, 2.6% C) and the results are shown schematically below the relevant fraction. The distance migrated by each protein band (R_m) was measured relative to the dye front and an included protein marker (ovalbumin). Band intensities (protein stain) are represented by the width of the bands in the diagram. Pooled fractions are denoted by the horizontal bars. (b) After equilibration of the column with 50 mM-Tris/HCl buffer, pH 7.8, containing 2-mercaptoethanol (0.1 mM) and 50 mM-NaCl, the enzyme was eluted with a linear gradient (350 ml) of 0–10 mM-chloramphenicol in the same buffer. The flow rate and fractions were as in (a). Selected fractions were analysed as in (a), but with gels of slightly different composition (7.5% T, 2.6% C), to optimize the resolution of β_4 and $\alpha\beta_3$ species of chloramphenicol acetyltransferase. The R_m values therefore do not correlate with those observed in (a).

Table 2. Partial purification of chloramphenicol acetyltransferase variants from *E. coli* J53 (R429; R387) by affinity chromatography

Buffer was 50 mM-Tris/HCl, pH 7.8, containing 2-mercaptoethanol (0.1 mM). Total overall recovery of chloramphenicol acetyltransferase at this stage was 51%. The crude extract applied to the affinity resin was the heat-treated and dialysed extract described in Table 1. Fractions DE1–DE4 represent pooled eluates taken for subsequent ion-exchange chromatography as described in Fig. 3.

Purification step	Volume (ml)	Chloramphenicol acetyltransferase (units/ml)	Total activity (units)	Yield (%)
Crude extract	375	79	29 520	
Enzyme bound to affinity resin	—	—	26 520	(100)
50 mM-buffer wash (A)	400	2.4	960	3.6
50 mM-buffer (A) with chloramphenicol (0–10 mM) gradient	—	—	DE1, DE2, DE3 11 112	42.0
50 mM-buffer wash containing 50 mM-NaCl (B)	600	1.0	600	2.2
50 mM-buffer (B) with chloramphenicol (0–10 mM) gradient	—	—	DE4 5500	21.0
50 mM-buffer wash containing 75 mM-NaCl (C)	370	1.7	633	2.4
50 mM-buffer (C) with chloramphenicol (0–10 mM) gradient	—	—	1700	6.4
			20 505	77.6

Fig. 3. Ion-exchange chromatography of chloramphenicol acetyltransferase variants on DEAE-cellulose

Pooled fractions (DE1–DE4) from the affinity-chromatography step were subjected to ion-exchange chromatography at 4°C on DE52 DEAE-cellulose resin (100 ml; 20 cm × 2.5 cm) equilibrated with 50 mM-Tris/HCl buffer, pH 7.8, containing 0.1 mM-2-mercaptoethanol and 0.2 mM-chloramphenicol. Chloramphenicol acetyltransferase was eluted with a linear gradient (1500 ml) of 0.1–0.3 M-NaCl in equilibrating buffer. This is shown for the profile for fraction DE1 only; it applies equally for the remaining profiles. The column flow rates were as follows: DE1, 40 ml/h; DE2, 30 ml/h; DE3, 22 ml/h; DE4, 27 ml/h. In all cases 10 ml fractions were collected and the enzymic species present in peak fractions were identified by non-denaturing gel electrophoresis.

yielded electrophoretically homogeneous preparations of α_4 , $\alpha_3\beta$, $\alpha_2\beta_2$ and β_4 variants of chloramphenicol acetyltransferase (Fig. 4). The overall yield of the hybrid preparations was 1140 units ($\alpha_3\beta$) and 1940 units ($\alpha_2\beta_2$).

Fig. 4. Non-denaturing electrophoresis of purified chloramphenicol acetyltransferase variants from *E. coli* J53 (R429; R387)

Electrophoresis of parental (α_4 and β_4) and hybrid ($\alpha_3\beta$ and $\alpha_2\beta_2$) chloramphenicol acetyltransferases (20–30 μ g each) was performed in polyacrylamide gels (8.0% T, 2.6% C) under non-denaturing conditions. Ovalbumin (20 μ g) was included in each sample as an internal reference. Gels (1)–(4) contain the isolated species; gel 5 contains a mixture of the isolated species. The apparently poor band sharpness of the β_4 species relative to the α -containing species is a typical and reproducible feature of most type-I homotetramers and may represent microheterogeneity or variable masking of charged residues.

The chromatographic behaviour of each hybrid variant was intermediate between those of the parental enzymes. The latter are anomalous in that, on the basis of the electrophoretic mobility of each variant, elution from the DEAE-cellulose DE52 resin was expected in the reverse order to that observed. This phenomenon is reproducible, but has not been studied further. Its explanation probably lies in the interaction of each of the enzyme variants with cellulose or ethyl-cellulose under the conditions employed. Although the type-III enzyme (α_4) has the highest net negative charge as deduced from the electrophoretic results, it is known to be more readily eluted from hydrophobic supports than the type-I (β_4) variant (Zaidenzaig & Shaw, 1976).

Determination of the subunit composition of hybrid chloramphenicol acetyltransferase by amino acid analysis

Although the purified hybrid chloramphenicol acetyltransferases were identified tentatively as $\alpha_3\beta$ and $\alpha_2\beta_2$ with regard to subunit composition, it was necessary to confirm this presumptive assignment. From the amino acid composition of the α_4 and β_4 species of chloramphenicol acetyltransferase (Packman, 1978), it is possible to compute and predict the ratio of aspartic acid to glutamic acid expected for any enzyme comprising any combination of α and β polypeptide chains in a tetrameric association. The observed (duplicate samples) and predicted ratios were compared for parental and hybrid enzymes (Table 3).

The data for the $\alpha_2\beta_2$ hybrid species support the presumptive assignment of subunit composition. The data for the $\alpha_3\beta$ species are less conclusive and the assignment of the subunit composition to this species of enzyme therefore remains tentative.

Several other observations suggest, however, that the $\alpha_3\beta$ composition is probably correct. The α and β chains are separable by SDS/polyacrylamide-gel electrophoresis, having apparent molecular weights of 24 500 and 24 000 respectively. Although the two chains each bind dye to slightly different extents, it is

still possible by inspection to compare the putative $\alpha_3\beta$ sample alongside the polypeptides of $\alpha_2\beta_2$ and the parental enzyme. Such a comparison strongly supports the $\alpha_3\beta$ composition proposed for the asymmetric heteromer (results not shown).

Determination of the specific-activity values of hybrid chloramphenicol acetyltransferases

The specific activity of each enzyme variant was determined by relating the observed catalytic activity of each enzyme (measured under identical and standard assay conditions) to its protein content, as measured by amino acid analysis.

Parental variants demonstrated specific activities of 595 units/mg (α_4) and 203 units/mg (β_4). These values are in good agreement with those previously determined for the purified enzymes isolated from *E. coli* strains J53 (R387) and J53 (R429), 570 and 195 units/mg respectively (Packman & Shaw, 1981).

The specific activity of the $\alpha_2\beta_2$ hybrid was 293 units/mg and that of the $\alpha_3\beta$ hybrid was 524 units/mg. In a model which allows each subunit to function independently, values of 500 and 400 units/mg would have been expected for the specific activities of the $\alpha_3\beta$ and $\alpha_2\beta_2$ hybrids respectively. Whereas the measured specific activity for the $\alpha_3\beta$ enzyme approaches the predicted value for an independent or non-co-operative model, that for the $\alpha_2\beta_2$ enzyme does not. The data at present allow more in the way of speculation than explanation. A satisfactory model must await a detailed kinetic analysis of the catalytic activity of the $\alpha_2\beta_2$ variant in particular and the use of genetic and immunochemical approaches to dissect the contributions of each polypeptide component.

Identification of the 'buried' lysine residues in chloramphenicol acetyltransferase hybrids

The buried lysine residues in the naturally occurring chloramphenicol acetyltransferase variants specified by plasmids R429 and R387 have been identified (Packman & Shaw, 1981). In the type-I chloramphenicol acetyltransferase (β_4)

Table 3. *Determination of the subunit composition of hybrid chloramphenicol acetyltransferase by amino acid analysis*
Values for Asx and Glx that are underlined were determined experimentally; the remaining values are derived from these data by computation.

Enzyme subunit composition	Expected average composition (mol/mol of subunit)		Asx/Glx ratio	Observed value
	Asx	Glx		
α_4	<u>27.04</u>	<u>20.49</u>	1.32	1.32, 1.33
$\alpha_3\beta$	25.28	21.05	1.20	1.12, 1.13
$\alpha_2\beta_2$	23.52	21.61	1.08	1.05, 1.06
$\alpha\beta_3$	21.76	22.60	0.96	—
β_4	<u>20.00</u>	<u>22.73</u>	0.88	0.88, 0.89

specified by plasmid R429, only lysine-136 was observed to be inaccessible to modification with methyl acetimidate in the native tetramer. The experimental design involved amidination with non-radioactive methyl acetimidate followed by treatment with methyl [1- 14 C]acetimidate under denaturing conditions to yield the uniquely labelled amino group of lysine-136. Analysis by electrophoresis and chromatography of peptides generated by proteolysis of such protein yielded two radioactive peptides, designated IR-1 and IR-2. Peptide IR-1 was shown to have the sequence Phe-Pro-Lys¹³⁶-Gly-Phe, whereas peptide IR-2 was observed to be a partial digestion fragment having in addition 1 mol each of Ala, Leu and Tyr per mol.

Similar analysis of chloramphenicol acetyltransferase_{R387} (α_4) also yielded two radioactive peptides, IP-1 and IP-2. Peptide IP-2 has the sequence Thr-Ser-Lys³⁸-Ile-Asp-Ile-Thr-Leu. Peptide IP-1 contains Ser-Leu in addition, at the N-terminus. The 'buried' lysine group is at position 38 in the type-III polypeptide chain (α) specified by plasmid R387.

Since the α_4 and β_4 variants show extensive amino acid sequence homology, it is possible and likely that, if lysine-136 (β) and lysine-38 (α) serve similar functions in stabilizing tertiary or quaternary structure of the enzyme, these two regions of the polypeptide chain may be close to each other in the native enzyme.

The involvement of lysine-136 (β) and lysine-38 (α) as determinants of subunit interactions rests on circumstantial evidence and discounts the alternative view that the data are compatible with an intrasubunit location of the buried residues. Examination of the buried lysine groups in a hybrid tetramer composed of α and β subunits is of interest, since in such hybrids α/β interfaces must occur; the opportunity exists therefore to compare the reactivity of functional groups at the contact regions of an heteromeric dimer and to inquire whether they are similar to or different from those in the homomeric dimer, depending upon the homology and complementarity of the subunits in each case. The fact that hybrid forms of chloramphenicol acetyltransferase may be isolated is evidence of stable heteromeric interaction, but raises at the same time the question of whether lysine-136 (β) and lysine-38 (α) each remain as inaccessible to solvent in the hybrid enzyme as they do in the parental (β_4 and α_4) tetramers. The studies described below suggest that they do not.

If the same two relevant lysine residues are buried in the hybrid chloramphenicol acetyltransferases as in the parental molecules, no inference can be drawn as to the topographic location of these residues. If different lysine residues are buried in the hybrid enzymes, an intersubunit location is favoured and an hypothesis may be formulated about the nature of

the subunit interactions. At the outset, however, there is a potential drawback to this approach when dealing with symmetrical tetramers ($\alpha_2\beta_2$). When in such hybrids there are heterologous subunit interactions, a single type of binding domain is assured (Hajdu *et al.*, 1976). If, however, isologous interactions ('dimer of dimers') are present, two distinct types of subunit interactions may occur; one between the subunits of a given dimer and the other between the dimers themselves. Thus, if lysine-136 (β) and lysine-38 (α) are involved in intersubunit binding, an isologous ($\alpha_2\beta_2$) hybrid might not contain heteromeric $\alpha\beta$ interactions. That is, its subunit arrangement may be α_2/β_2 rather than $\alpha\beta/\alpha\beta$, where '/' denotes the dimer-dimer interface. The lysines in each case could be located at the interfaces between the two subunits of a homomeric dimer. Only the $\alpha\beta$ dimer would be certain to yield the desired contacts between dissimilar subunits. Such a situation is assured in an asymmetric ($\alpha_3\beta$ or $\alpha\beta_3$) hybrid.

Notwithstanding the potential problems outlined above and the technical difficulties in producing unambiguous chemical-modification data, the approach offered an opportunity to explore the general method and gain useful information on the quaternary structure of chloramphenicol acetyltransferase in advance of the results of X-ray-diffraction studies.

Each of five samples of chloramphenicol acetyltransferase variants was amidinated by two additions of methyl acetimidate (each to give 50 mM final concn.) as described in the Materials and methods section. The samples for amidination consisted of the following enzymes: α_4 , 1.7 mg; $\alpha_3\beta$, 1.5 mg; $\alpha_2\beta_2$, 3.5 mg; β_4 , 2.0 mg; and a mixture of α_4 (1.0 mg) and β_4 (1.0 mg). After isolation of the amidinated tetrameric material in each experiment, the proteins were unfolded in 8 M-guanidinium chloride and labelled with methyl [1- 14 C]acetamidate.

The incorporation of radioactivity from the 14 C-labelled imidoester into the amidinated protein is shown in Table 4. Approx. 1 mol of radioactive reagent per mol of subunit was incorporated into each enzymic species. The two-dimensional thin-layer analysis of the peptides generated by proteolysis of each protein sample yielded the radioautographs shown schematically in Fig. 5.

The peptide 'maps' for each of the α_4 - and β_4 -derived samples were as previously observed (Packman & Shaw, 1981), and that derived from an equimolar mixture of α_4 and β_4 variants confirms the reproducible migration of the radiolabelled peptides in the presence of peptides derived from the complementary protein. Similarly there is no evidence by gel electrophoresis under non-denaturing conditions for the formation of hybrid

tetramers on incubation of a mixture of the native or chemically modified α_4 and β_4 enzymes under the conditions employed in this experiment (results not shown).

The 'map' for species $\alpha_2\beta_2$ is clearly different from that for the ($\alpha_4 + \beta_4$) mixture. Although peptides IR-1 and IR-2 are present in both samples, the α -chain-derived peptides IP-1 and IP-2 are no longer radioactively labelled in the sample from the modified $\alpha_2\beta_2$ hybrid. Peptide IP-2 has been 'replaced' by peptide IP-3. The decapeptide IP-1 (a proteolytic 'precursor' of octapeptide IP-2) is thus

likely to have been replaced by a radioactive peptide of similar mobility, which is in turn related in sequence to peptide IP-3.

The peptide 'map' from the $\alpha_3\beta$ enzyme was difficult to interpret. Although the radioactive neutral peptides were less discretely separated than those in the parallel experiments, a careful examination of the radioautogram revealed the presence of three closely migrating peptides with chromatographic mobilities corresponding to peptides IP-1, IP-2 and IP-3. The summed intensity of the three neutral peptides was clearly greater than that of the two basic peptides (IR-1 and IR-2), suggesting that all of the neutral peptides were derived from the α polypeptide chain.

The conclusion from these experiments is that the availability of lysine-38 (α) to chemical modification is governed to a significant degree by the structure of the subunits with which the α -subunit is in contact. In the $\alpha_2\beta_2$ hybrid, lysine-38 (α) is not buried (peptides IP-1 and IP-2 absent), whereas lysine-136 (β) remains so (peptides IR-1 and IR-2 present). We conclude that a previously exposed lysine residue of the α polypeptide is now buried in the $\alpha_2\beta_2$ hybrid, giving rise to the radioactive peptide IP-3, which is formed after amidination with methyl [^{14}C]acetimidate in 8M-guanidinium chloride. It was not possible to determine directly the amino acid sequence of

Table 4. Amidination of chloramphenicol acetyltransferase variants under denaturing conditions with methyl [^{14}C]acetimidate

The values in parentheses are those obtained previously from these variants isolated from single-plasmid strains of *E. coli* (Packman & Shaw, 1981). They are included here for comparison.

Enzymic species	Incorporation of ^{14}C (mol/mol of subunit)
α_4	1.02 (1.10)
$\alpha_3\beta$	1.19
$\alpha_2\beta_2$	1.32
β_4	1.23 (1.28)

Fig. 5. Radioautographs of the peptide 'maps' derived from proteolysis of parental and hybrid chloramphenicol acetyltransferase

Parental (α_4 and β_4 homotetramers and an $\alpha_4 + \beta_4$ mixture) and hybrid ($\alpha_3\beta$ and $\alpha_2\beta_2$) chloramphenicol acetyltransferase variants were amidinated with methyl acetimidate under native conditions. After unfolding in 8M-guanidinium chloride, the proteins were labelled with methyl [^{14}C]acetimidate, *S*-carboxymethylated and digested as described in the text. The resulting peptides were analysed by electrophoresis (pH 6.5) and chromatography (butan-1-ol/acetic acid/water/pyridine, 15:3:12:10, by vol.) on silica gel. Fluorescent markers are represented by open circles; radioactive peptides are represented by closed circles. No attempt has been made to represent relative intensities of these peptides, the importance of which is discussed, where relevant, in the text. The relative radioautographic intensities of the neutral and basic peptides in the $\alpha_2\beta_2$ -species 'map' were equal, whereas on the 'map' for the $\alpha_3\beta$ species those of the neutral peptides were approx. 3-fold more intense than the basic peptides, and peptide IP-3 was less intensely radioactive than IP-2. See the text for an explanation of the identities of peptides IP-1, IP-2, IR-1 and IR-2, which are described more fully in Packman & Shaw (1981). The dotted vertical line in each radioautograph indicates the location of neutral peptides.

Fig. 6. Models for the subunit interactions of hybrid chloramphenicol acetyltransferase

Four models for the subunit interactions of chloramphenicol acetyltransferase are examined. Two are based on an isologous arrangement of subunits in the $\alpha_2\beta_2$ hybrid and two on a heterologous arrangement. In the isologous models, the interface between the dimers is denoted by a double line, and we have assumed that there are no lysine interactions across this interface. The relative orientation of the two dimers will therefore have no influence on the lysine residues buried. In the heterologous models a simple cyclic arrangement of subunits is considered. Each model is constructed by using two requirements derived from the experimental results described in the text: (1) lysine-38 (α) is buried only at an $\alpha\alpha$ interface and (2) the lysine residue of peptide IP-3 is buried only at an $\alpha\beta$ interface. All models are then examined for the number and identity of buried lysine residues expected from each arrangement of two α and two β subunits. Comparison of the expected results with the observed data for the buried lysine residues in the $\alpha_2\beta_2$ hybrid allows (a) and (c) to be ruled out as candidates for the subunit interactions of chloramphenicol acetyltransferase.

peptide IP-3 because of the very small amounts of material available. Studies are required to characterize IP-3 indirectly by co-chromatography with proteolytic fragments of known sequence from fully amidinated chloramphenicol acetyltransferase_{R387}.

In the $\alpha_3\beta$ hybrid, both peptides IP-3 and IP-2 are radioactively labelled, the latter to a higher specific radioactivity if the yields of the two peptides are similar. If this assumption is correct, then the results are compatible with labelling of two of the α -subunits at lysine-38 and the third at some other position.

A model for the subunit interactions of chloramphenicol acetyltransferase

Taken together, the radioautographs derived from the hybrids suggest that the lysine residue of peptide IP-3 of the α -subunit becomes inaccessible to chemical modification only when the α -subunit is in association with a β subunit, but not in a homomeric interaction. Our interpretation of the results in relation to subunit interactions is necessarily limited in the absence of independent evidence bearing on the symmetry of chloramphenicol acetyltransferase. We can infer that lysine-38 (α) and lysine-136 (β) are likely to be located at contact regions between

subunits and that the lysine residue of peptide IP-3 of the α -subunit is buried at an $\alpha\beta$ interface rather than an $\alpha\alpha$ interface.

Important implications arise from this tentative conclusion when we consider possible models for the subunit arrangement of chloramphenicol acetyltransferase based on the isologous and heterologous interactions of four subunits. Considering first an isologous hybrid ($\alpha_2\beta_2$) tetramer, we infer from the above data that the quaternary structure of the oligomer may involve a tetramer composed of two heteromeric dimers ($\alpha\beta/\alpha\beta$) rather than two homomeric dimers ($\alpha\alpha/\beta\beta$). This is shown diagrammatically in Figs. 6(a) and 6(b).

On the basis of a heterologous association, the data are also compatible with one of two possible models (Figs. 6c and 6d). Since lysine-38 (α) is buried at $\alpha\alpha$ interfaces, whereas the lysine of peptide IP-3 is buried at $\alpha\beta$ interfaces, a model based on a heterologous association of two α and two β subunits must preclude an $\alpha\alpha$ binding region. We can therefore rule out the model in Fig. 6(c) as a candidate for the subunit arrangements of chloramphenicol acetyltransferase. A choice between the two remaining models (Figs. 6b and 6d) must await

definitive data bearing on the symmetry of the tetramer.

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