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Hybridization of Variants of Chloramphenicol Acetyltransferase Specified by *fi*$^+$ and *fi*$^-$ R Factors

(episomes/enzyme evolution/antibiotic resistance/plasmids/protein subunits)

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ABSTRACT **An *fi*$^-$ type of R factor for transferable chloramphenicol resistance carries the structural gene for a novel species of chloramphenicol acetyltransferase (EC 2.3.1.99). The enzyme associated with the *fi*$^-$ plasmid is distinct from that described for *fi*$^+$ R factors. The *fi*$^+$- and *fi*$^-$-related variants of chloramphenicol acetyltransferase were hybridized *in vivo* and *in vitro*. Both techniques yielded only a single symmetrical heteromeric species rather than the three (A_3B_1, A_2B_2, and A_1B_3) hybrids predicted from the tetrameric (identical monomers) structure of chloramphenicol acetyltransferase.**

Enzymatic inactivation of chloramphenicol (CM) is the prevalent biochemical mechanism responsible for CM resistance mediated by episomal genes (R factors) in enteric bacteria (1, 2). An analogous extrachromosomal element ("plasmid") carries the structural gene for a similar catalytic protein in CM-resistant staphylococci, but the kinetic and physical properties of the gram-negative (R factor) and staphylococcal (plasmid) types of chloramphenicol acetyltransferase (CAT; EC 2.3.1.99) are quite distinct (3–5). Although both enzymes catalyze the acetyl-coenzyme A-dependent O-acetylation of CM, the enteric (R factor) form of CAT is produced constitutively whereas synthesis of the staphylococcal enzyme and the presence of the associated CM-resistant phenotype are induced by CM and certain related isomers and analogues (3–5).

The ease with which CAT can be assayed and purified (3), and the availability of a simple histochemical stain for identification of enzyme activity of polyacrylamide gels (3) suggested a search for diverse electrophoretic variants of CAT. At least four naturally occurring electrophoretic variants of the staphylococcal type of CAT have been observed (ref. 8; and Sands, L. & Shaw, W. V., manuscript in preparation), but, until recently, only one "wild type" electrophoretic type of CAT has been observed among clinical isolates of CM-resistant, R-factor-bearing enteric bacteria. This observation can be contrasted with the relative ease with which *in vitro* mutagenesis of such strains yields forms of CAT with catalytic stability or electrophoretic properties distinct from those of the parental enzyme (6–9).

While all naturally occurring forms of R-factor CAT have shown an apparent identity in net charge, it is significant that all CAT-containing (CM-resistant) strains examined have carried the *fi*$^+$ type of R factor, so named for the ability of such drug-resistant plasmids to inhibit the fertility of host strains also harboring the sex factor (F). Watanabe *et al.* (10) called attention to the curious absence of CM resistance among *fi*$^-$ factors. Exceptions to this generalization have been reported more recently (11–14).

The experiments to be described were prompted by the lack of information on the biochemical mechanism of CM-resistance determined by *fi*$^-$ R factors. Preliminary studies of six such isolates have revealed (*a*) sufficient CAT activity in all strains to account for their resistance to CM, and (*b*) several novel types of CAT that are immunologically and catalytically different from the more common (*fi*$^+$) enzyme (Foster, T. J. and W. V. Shaw; manuscript in preparation). One of the *fi*$^-$ R factors examined yielded an enzyme with a strikingly different electrophoretic mobility as compared to the *fi*$^+$ type of CAT, and the present report deals with the purification and properties of this *fi*$^-$ enzyme as well as unanticipated results obtained after hybridization of the *fi*$^-$ and *fi*$^+$ forms of CAT *in vivo* and *in vitro*.

MATERIALS AND METHODS

The host strain of *Escherichia coli* for all studies is J53 of Clowes and Hayes (15), which is F$^-$, *pro*$^-$, *met*$^-$, and *lac*$^+$. R387 is an R factor (*fi*$^-$) from a strain of *Shigella flexneri* isolated in Roumania; it confers resistance to streptomycin and chloramphenicol. R429 came from a recent isolate of *Klebsiella sp.* at Hammersmith Hospital and is of the *fi*$^+$ type, bearing resistance to ampicillin, tetracycline, chloramphenicol, and kanamycin. Both R factors were transferred by conjugation to strain J53 (16). Factors R387 and R429 did not exclude one another and coexisted stably in strain J53. The *fi*$^-$ factor (R387) belongs to a compatability group distinct from those recently described (14). Each R factor was independently transmissable from the "double" R-factor strain.

Chloramphenicol acetyltransferase (CAT) activity was determined spectrophotometrically (3) and was purified as described (3), except that the alumina-gel absorption step was omitted before ion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose. Analytical polyacrylamide disc gel electrophoresis was performed as described for native CAT (3), with both the Amido Black stain for protein and the coupled tetrazolium reaction for enzyme activity. The method of Weber and Osborn (17) was used for determination of the apparent molecular weights of the monomeric (inactive) form of CAT (5). Isoelectric focusing of purified CAT from the *fi*$^+$/*fi*$^-$ dual R factor strain was accomplished as described

Abbreviations: CAT, chloramphenicol acetyltransferase; CM, chloramphenicol.

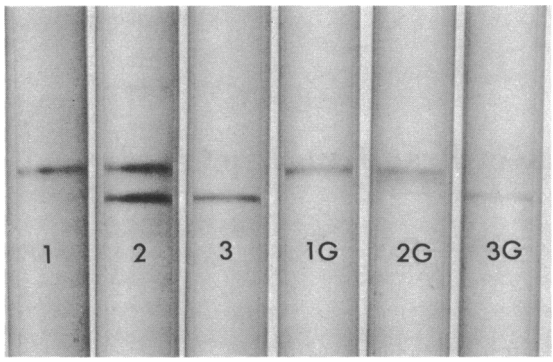

FIG. 1. Polyacrylamide gel electrophoresis of CAT. Gels *1* and *3* contained 50 μg each of purified native enzyme from R429 (fi^+) and R387 (fi^-), respectively, while gel *2* shows the results obtained when equal amounts of both were mixed immediately before electrophoresis. Gels *1G*, *2G*, and *3G* demonstrate the results obtained after reversible denaturation of the two species of CAT in the presence of guanidine hydrochloride and mercaptoethanol (see *Methods*). After removal of the denaturing reagents by dialysis, the fi^+-associated (*1G*) and fi^- (*3G*) forms of CAT migrated with mobilities characteristic of untreated native enzymes, whereas gel *2G* shows the third (intermediate) species obtained when guanidine dissociation was performed on a mixture of the purified enzymes. The faint bands above and below CAT in gels *1*, *2*, and *3* failed to show CAT activity with the histochemical stain. Migration was *downward* toward the anode.

(19), with the LKB apparatus and the pH 3–6 ampholyte reagent. Molecular weights of CAT subunits derived from the fi^+ (R429)- and fi^- (R387)-associated enzymes were determined (18). Samples were reduced and dissociated with 2-mercaptoethanol (0.1 M) in the presence of 6 M guanidine·HCl for 4 hr at room temperature before application to a 1.5 × 25 cm column of Agarose (Bio-Gel A-5M). Elution profiles of CAT and marker proteins (cytochrome *c*, ovalbumin, and myoglobin) were developed with the above solvent, and proteins were determined by turbidity at 450 nm after treatment of each sample (0.3 ml) with 1.2 ml of 4% trichloroacetic acid. The void volume of the column was 5.2 ml, as determined with Dextran Blue (Pharmacia).

Similar immunologic methods to those previously cited were used for both precipitin tests (Ouchterlony) and neutralization studies (3–5). The antiserum was prepared by immunization of goats with three intramuscular injections (5 mg each) of CAT in Freund's complete adjuvant, biweekly. The serum of each goat before immunization was used as a control and for dilution of specific antisera to CAT (see Fig. 4).

RESULTS

Activity of CAT in Cell Extracts. Preliminary experiments revealed that the strain bearing the fi^- R factor (R387) produced CAT. Furthermore, the specific activity of CAT (1.0 units/mg) from the dual plasmid strain equalled the sum of the individual contributions of the fi^+ (0.6 units/mg) and fi^- (0.4 units/mg) R factors when the latter were measured independently. The use of CM-dependent phenazine/tetrazolium stain for free CoASH release (see *Methods*) revealed that the electrophoretic mobilities of the "conventional" (fi^+) and the R387 (fi^-) types of CAT were quite distinct, the latter showing a substantially greater net negative charge at pH 8.5. Since the resolution of the histochemical stain was not adequate to quantitate the contributions of the fi^+ and fi^- enzymes to the activity of the double R factor extract, it was necessary to purify the CAT activity from each of the three strains before further studies. CAT was purified from the fi^+ (R429) and fi^- (R387) strains as described (3) and yielded for each a protein fraction that was homogeneous by disc gel electrophoresis. The two species were easily distinguished and migrated independently with characteristic relative mobilities when mixed before electrophoresis (Fig. 1).

Purification and Characterization of CAT from fi^+/fi^- Strain. The apparent independent and additive contributions of each plasmid to the specific activity of CAT in the dual plasmid strain and the knowledge that native R-factor CAT exists as a tetramer of four identical subunits (5, 8) raised several questions concerning the molecular forms of CAT in the fi^+/fi^- derivative. By analogy with other genetic systems (see *Discussion*) the assumption was made that two extreme possibilities might be anticipated. If the *in vivo* synthesis of CAT in the diploid strain yielded two types of monomeric subunits that were sufficiently different that hybridization was impossible, then only the two parental (fi^+/ and fi^-) species of CAT would be found. An alternative hypothesis presupposed that sufficient homologies might exist between the fi^+ and fi^- species of CAT monomers that a "family" of five native forms would result from random hybridization of the heteromeric subunits (A_4, A_3B_1, A_2B_2, A_1B_3, and B_4). To decide between these and other more complicated possibilities, it was necessary to purify CAT from the fi^+/fi^- strain. Conventional techniques

TABLE 1. *Purification of CAT from dual plasmid (fi^+/fi^-) strain*

Purification step	Protein mg total	Enzyme activity units total	Specific activity
1. Crude extract	10,450	10,010	0.96
2. Streptomycin sulfate	5,735	7,955	1.39
3. Heat	1,350	8,460	6.29
4. Ammonium sulfate	845	7,150	8.47
5. DEAE chromatography (1) (pooled peak fractions)	245	3,140	12.8
6. G-100 Sephadex (pooled peak fractions)	68	2,770	40.7
7. DEAE chromatography (2)*			

The yield of bacterial cells from 14 liters of *E. coli* J 53 (R429/R387) in Penassay broth was 39.9 g (wet weight). CAT was purified as described (3) after cell disruption with a motor-driven French pressure cell (Aminco) operating at 20,000 lb/in². The first DEAE chromatography step was performed on a 2.5 × 40 cm column of DE-52 microgranular DEAE (Reeve-Angel) with a 1-liter linear gradient of NaCl (0–0.4 M) in 10 mM Tris·HCl (pH 7.8) containing CM (5 mM) and 2-mercaptoethanol (5 mM). The pooled "peak" eluted at 0.13–0.19 M NaCl was concentrated to 10 ml by ultrafiltration and applied to a 2.5 × 100 cm column of Sephadex G-100 operating upwards with the above Tris–CM–mercaptoethanol buffer containing 0.2 M NaCl. A sharp and symmetrical peak of CAT was obtained with more than 80% of the applied activity obtained in four fractions of 5.5 ml each.

* See Fig. 2.

(see *Methods*) were used with the results as summarized in Table 1.

Prior calibration of the Sephadex G-100 column used in the final purification step confirmed that the elution volume for enzyme activity was consistent with the expected molecular weight of 80,000 for native CAT (8). Since no other peaks of CAT activity were observed, we concluded that all active (tetrameric) species of purified CAT shared the same apparent molecular weight. Disc gel electrophoresis of the Sephadex G-100 peak revealed a broad band of enzyme activity with the histochemical stain and three discrete protein bands in the region of activity when a parallel gel sample was stained with Amido Black.

Thus, the purification of CAT activity from the fi^+/fi^- "hetero-R" strain had yielded (*a*) the familiar fi^+-associated CAT, (*b*) the CAT species typical of R387 (fi^-), and (*c*) a third protein component with CAT activity that possessed intermediate electrophoretic mobility. In order to separate the three apparent species of CAT, we passed the pooled Sephadex G-100 peak through a final DEAE column using a shallow (0.1–0.3 M) NaCl gradient. In contrast to the earlier ion-exchange chromatography step, the final DEAE column yielded three distinct and equal peaks of CAT activity, which accounted for 96% of the enzyme activity applied. When each fraction was examined by disc gel electrophoresis followed by staining for CAT activity, the fi^--associated species of CAT was eluted first and the fi^+ type last. Fig. 2 shows the parallel findings when gels were stained for protein. Both methods were consistent with the conclusion that a new species of CAT had been synthesized *in vivo* that had net charge properties intermediate between the parental fi^+ and fi^- enzyme species, suggesting that the novel form of CAT might be a hybrid of A_2B_2 composition. Isoelectric focusing of the pooled Sephadex G-100 peak yielded results that complemented the electrophoretic data in that apparent isoelectric points of pH 4.8, 5.1, and 5.4 were observed for the fi^-, "hybrid," and fi^+ enzymes, respectively.

To rule out the possibility of discrimination against the "missing" A_3B_1 and A_1B_3 species of CAT by the purification procedure, we applied a sample of crude enzyme from the dual plasmid strain to the same Sephadex G-100 column described for Step 6 (Table 1). All CAT activity appeared in a sharp peak that yielded only the three enzymatically active electrophoretic variants seen in Fig. 2.

In Vitro Hybridization of fi^+- and fi^--Associated Species of CAT. To interpret the results of the *in vivo* studies from the point of view of direct interactions between homologous but nonidentical subunits, we tried to hybridize the fi^+ and fi^- enzymes *in vitro* after dissociation of the parental enzymes in 6 M guanidine·HCl. Fig. 1 contrasts the electrophoretic mobilities of the parental enzymes in the absence of prior dissociation with the behavior of each after reversible denaturation. When either the fi^+- or the fi^--associated forms of CAT are denatured and allowed to reassociate in the absence of their counterparts, the mobility of parental (native) CAT is observed. Gel *2G* in Fig. 1 depicts the products of reversible denaturation when both the fi^+ and fi^- species of CAT are present throughout the procedure. The three bands observed are identical with the results of electrophoresis of the final Sephadex G-100 "peak" (Table 1) and colinear with each of the bands demonstrated in Fig. 2. The above results would predict that reversible denaturation of purified hybrid CAT would yield an electrophoretic pattern showing all three forms due to random reassociation of the heteromeric dimers, which appear to be obligatory intermediates in the formation of native CAT tetramer. In point of fact, when tube 46 of the final DEAE column was denatured reversibly in identical fashion to the purified parental enzymes, the results were indistinguishable from those shown in gel *2G* of Fig. 1.

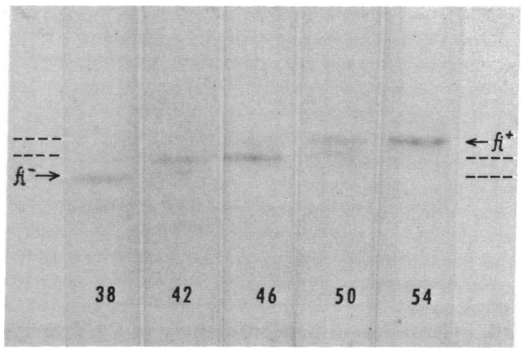

FIG. 2. Polyacrylamide gel electrophoresis of fractions from the final DEAE column after staining for protein with Amido Black. Aliquots containing about 0.5 unit of CAT were taken from the indicated fractions. The peak tubes for the fi^-, hybrid, and fi^+ enzymes were *38*, *46*, and *54*, respectively. Duplicate gels were also stained for CAT activity and yielded identical results. The faint protein bands below those of the three CAT variants were devoid of enzyme activity and were judged to be contaminants.

Interpretation of the above studies rested on the basic premise that the quaternary structure of both the fi^+- and fi^--associated forms of CAT are homologous and that each parental species consists of four identical subunits. The cochromatography of all three species on a calibrated column of Sephadex G-100 confirmed that the native forms of CAT had identical molecular weights of 80,000. Electrophoresis of both parental enzymes in sodium dodecyl sulfate (17) indicated that the fi^+ and fi^--associated subunits are identical and equal to 20,000 when analyzed against standards previously used (5). Furthermore, the parental types of CAT are, in fact, identical in charge as well as size since, after reduction and alkylation of the purified fi^+ or fi^- species of CAT, only one polypeptide is seen when electrophoresis is performed in 8 M urea.

Although the results obtained from *in vivo* hybridization of the fi^+- and fi^--associated types of CAT are consistent with a nonrandom association of the monomeric (20,000) subunits, the *in vitro* studies are subject to the criticism that 6 M guanidine·HCl treatment failed to dissociate the enzyme beyond the dimer stage (40,000). Reassociation under such circumstances would be expected to yield only the observed A_2, A_2B_2, and B_4 species. To rule out such an interpretation, we mixed the purified fi^+ and fi^- types of CAT, reduced them with 0.1 M mercaptoethanol in the presence of 6 M guanidine·HCl, and analyzed them for molecular size on agarose gel columns (18). A single peak of protein was observed at an elution volume corresponding to an apparent molecular weight of 20,000, and reassociation after dialysis yielded active CAT with the three electrophoretic components seen in Fig. 1 (gel *2G*).

Immunological Analysis of Native and Hybrid Forms of CAT. Previous studies have shown that specific antibody to a given

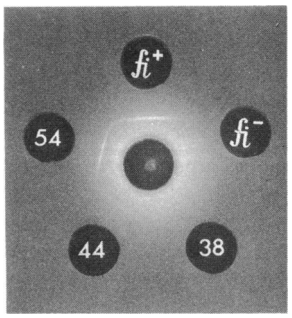

FIG. 3. Gel diffusion precipitin study of CAT variants. The *outer wells* contained 25 μg each of purified CAT from strains R429 (fi^+), and R387 (fi^-), and the appropriate DEAE fractions (Fig. 2) containing the corresponding native enzymes from the hybrid (R429/R387) strain. The *center well* contained 10 μl of goat antiserum prepared against the fi^+ type of CAT. The photograph of an unstained agar plate was taken after 24 hr incubation at room temperature. Preimmunization sera showed no precipitin lines with any of the preparations tested.

species of native CAT can neutralize and precipitate certain closely related but nonidentical forms (3, 5, 8). The availability of a specific antiserum for the fi^+-associated enzyme offered an opportunity to compare the fi^- (R387) form of CAT with conventional fi^+ (R429) species and to examine the reactivity of the observed fi^+/fi^- hybrid enzyme. Fig. 3 illustrates the results of a double diffusion (Ouchterlony) experiment comparing the reactivity of purified parental enzymes with fractions from the final DEAE column (Table 1 and Fig. 2) containing essentially pure species of both parental types as well as the hybrid enzyme (tube *44*). When tested at equal protein (CAT) concentrations, lines of identity and equal intensity were observed between the purified fi^+ enzyme and its counterpart in fraction *54*. No precipitin line was observed with the purified fi^- enzyme or the fraction (tube *38*) corresponding to it. A line of identity, but of diminished intensity, was seen for the hybrid enzyme (fraction *44*), which showed no detectable fi^+ CAT when examined by electrophoresis. The lack of immunological reactivity of the fi^- enzyme and the diminished precipitin reaction of the hybrid protein suggested that a quantitative neutralization study might provide confirmatory evidence for the proposed A_2B_2 structure of the hybrid enzyme. Fig. 4 shows the results obtained when equivalent amounts of CAT protein from the fi^+, fi^-, and hybrid enzymes are titrated with antiserum against CAT (fi^+). As expected from the precipitin data, the fi^--associated enzyme is not inhibited, whereas residual fi^+-related activity decreases in approximate proportion to the quantity of antiserum added. A small fraction of the total activity persists even under conditions of extreme antibody excess (20).

The intermediate degree of precipitation (Fig. 3) and the 50% neutralization (Fig. 4) of hybrid (fi^+/fi^-) CAT suggest that antibody can combine only with that portion of the native heteromeric protein that is isologous with the fi^+ parental type and that no interference occurs with substrate binding or catalytic activity of the heterologous (fi^-) portion. The immunological results are, therefore, consistent with the hypothesis that the hybrid enzyme is a tetrameric protein of A_2B_2 structure. The extent of indifference (about 50%) of the hybrid enzyme to an excess of fi^+ CAT-directed antibody is also in keeping with the observation that there is one CM-binding site per subunit of native CAT protein (W. V. Shaw, unpublished experiments).

DISCUSSION

The present study was suggested by the observations that, although apparently rare, fi^- R-factor-bearing strains of CM-resistant enteric bacteria can be isolated from natural sources (11, 14). The transferable fi^- plasmid R387 was chosen for intensive study because it carries the structural gene for a novel species of CAT. The R387-associated enzyme is distinctly different from the prototype fi^+ species (R429). Although the reaction mechanisms in both cases appear to be identical and the two species of enzyme share a common quaternary structure consisting of four identical subunits each, the net charge of the native enzymes and their respective reduced and alkylated subunits are clearly different. The obvious differences in primary sequence are reflected in both substrate affinity for CM (K_m = 8.7 and 22 μM for R429 and R387, respectively) and immunologic behavior (Figs. 3 and 4). The extent to which the R387 type of CAT is homologous with the conventional (fi^+) enzyme will show how many mutational events separate the two classes of CAT.

The unanticipated results of the *in vivo* and *in vitro* hybridization experiments present certain conceptual problems in understanding the quaternary structure of native CAT. Taken together, the results of earlier studies (8) and the data presented here describe the catalytic native enzyme as a protein of 80,000 molecular weight (5.2 S) that can be readily dissociated into four identical monomeric subunits of 20,000 each. Based upon well-established precedents, illustrated by studies with the tetrameric structure of aldolase (21), hybridization of two variants of CAT would have been expected to yield a five-numbered set of enzymes containing three hybrid (heteromeric) species in addition to the homomeric parental types.

Although the relative or complete absence of expected asymmetric heteromers (A_1B_3 and A_3B_1) might in some in-

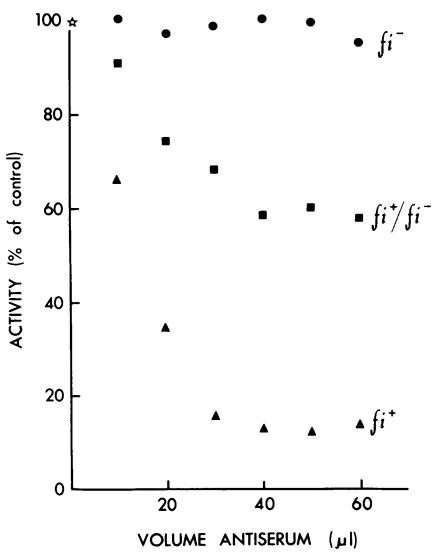

FIG. 4. Neutralization of CAT activity by antiserum. Fractions *38*, *44*, and *54* (see Fig. 2) were used as sources of the fi^-, hybrid, and fi^+ species of CAT as in Fig. 3. Each point represents a determination of residual enzyme activity after incubation of 5 μg of each enzyme with the indicated volumes of fi^+-specific antiserum against CAT for 40 min at 4°.

stances be explained by artifacts of the reversible dissociation methods used, the *in vivo* hybridization approach is less subject to such criticism. As Lew and Roth (22) have pointed out, there are certain theoretical limitations to the *in vivo* technique that uses bacterial strains carrying two distinguishable structural genes for the same oligomeric protein. One such objection, which also applies to the *in vitro* technique, is that the "missing" asymmetric hybrids are in fact formed but are unstable or enzymatically inactive. Such an interpretation seems unlikely in the present case since only three proteins are also seen after hybridization *in vitro* (Fig. 1). Similar reasoning can be advanced to counter an additional intriguing objection to nonrandom results obtained from *in vivo* hybridization; namely, a tendency for identical monomers to aggregate to dimers when present as neighboring nascent polypeptides on the same messenger RNA molecule (22).

Pending further studies, the conclusions from the existing hybridization data for CAT are that formation of the native enzyme from its four monomeric subunits proceeds by way of formation of the dimer, which must be composed of two identical or nearly identical polypeptides. We propose that aggregation of the dissimilar homomeric dimers is less critically dependent on homology, permitting formation of the observed symmetrical (A_2B_2) native tetramer.

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