Chloramphenicol Acetyl Transferase Formed by Wild-type and ComPLEMENTING R Factors in *Escherichia coli* K12

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SUMMARY

Chloramphenicol acetyl transferase (CAT) was purified from *Escherichia coli* K12 carrying R1 and 222 and from *E. coli* K12 *recA* (R1 Cm^s/R100-99Tc^rCm^s). The third enzyme has a lower affinity for chloramphenicol and greater heat lability than those specified by R1 and 222, compatible with the enzyme's resulting from interallelic complementation. CAT has a molecular weight of 80000, with subunits of 20000. Material cross reacting with anti-222 CAT serum was detected in Cm^s mutant cell sonicates.

INTRODUCTION

Transferable antibiotic-resistance (R) factors mediate resistance to chloramphenicol by determining the formation of chloramphenicol acetyl transferase (Shaw, 1966, 1967; Shaw & Brodsky, 1967; Suzuki & Okamoto, 1967; Mise & Suzuki, 1968). The enzyme purified from *Escherichia coli* R₆/K-10 has a molecular weight of 80000 and has four identical subunits (Shaw & Brodsky, 1968; Shaw, 1971).

We have isolated several chloramphenicol-sensitive (Cm^s) mutants of R1 that can recombine with each other in *rec*⁺ bacteria. Recombination experiments have also been carried out with these mutants, and mutants of R100-1, in *rec*⁻ bacteria (Foster & Howe, 1971). No Cm^r recombinants were formed in *recA* hosts, and the recombination frequency was reduced in *recB* and *recC* hosts.

Some of the R1 Cm^s mutants recombined with R100-1 Cm^s mutants in *rec*⁺ hosts, and Cm^r colonies were also formed when R1 Cm^s and R100-1 Cm^s mutants were jointly harboured by *recA* bacteria. These *recA* (R1 Cm^s/R100-1 Cm^s) hetero-R colonies grew slowly on agar containing chloramphenicol, and the presence of both R factors was found to be necessary for resistance to chloramphenicol to be maintained. The level of chloramphenicol resistance and the specific activity of the chloramphenicol acetyl transferase (CAT) formed were tenfold lower than those of wild-type Cm^r R factor-bearing cells. We have proposed that the properties of these cells result from interallelic complementation between Cm^s point mutants in the absence of recombination (Foster & Howe, 1971). Interallelic complementation, giving enzymes of low specific activity, has often been observed when different point mutants of a gene are introduced into the same cell (Fincham, 1966); it requires that the gene product be composed of two or more identical subunits, and is thought to result from partial conformational correction between two inactive mutant subunits. We now describe the purification and properties of the enzymes from wild-type and R1 Cm^s/R100-1 Cm^s cells, which provide further evidence for interallelic complementation.

METHODS

Nomenclature, materials, bacterial strains and R factors have been described (Foster & Howe, 1971). R100-99Tc^R is a Cm^S mutant of R100-1. R1 Ap^SCm^St⁻ mutants were maintained on minimal agar+kanamycin (40 µg/ml) and streptomycin (50 µg/ml). *Escherichia coli* *recA* (R1 Cm^S/R100-99Tc^R) was maintained on MacConkey agar+kanamycin (40 µg/ml), tetracycline (20 µg/ml) and chloramphenicol (10 µg/ml).

Chloramphenicol acetyl transferase (CAT) was purified from bacteria carrying R1, 222 and *recA* (R1 Cm^S/R100-99Tc^R) using a MSE continuous action rotor after growth at 37 °C for 24 h with aeration in nutrient broth (30 l). *recA* (R1 Cm^S/R100-99Tc^R) organisms were grown in kanamycin (100 µg/ml) and tetracycline (50 µg/ml) to maintain the hetero-R state. CAT was purified by the method used for R₆/K-10 (Shaw & Brodsky, 1968) with minor modifications. All steps were performed at 0 to 4 °C. Bacterial cells (wet weights: J5-3 (R1), 136 g; J5-3 (222), 112 g; *recA* (R1 Cm^S/R100-99Tc^R), 37 g) were suspended in standard buffer containing tris-HCl (10 mM), β-mercaptoethanol (0.5 mM) and chloramphenicol (0.2 mM) at pH 7.8. After sonic disruption bacterial debris was removed by centrifugation (30 000 g × 30 min). Streptomycin sulphate was added (1% final concentration) and the precipitate discarded after centrifugation.

Ammonium sulphate was added to 50% saturation and the precipitate dissolved in standard buffer. The resulting protein was heated (70 °C × 10 min) and quickly cooled. The precipitate was discarded after centrifuging, and ammonium sulphate was added to the supernatant to 50% saturation. The precipitate was dissolved in standard buffer (R1 and 222 CAT, 25 ml; *recA* (R1 Cm^S/R100-99Tc^R) CAT, 10 ml), which was exchanged for sodium phosphate (10 mM, pH 7.0) by dialysis. Alumina C_γ gel (Sigma, 0.7 mg wet weight/mg protein) was added to adsorb the enzyme, and after centrifuging 0.5 M sodium phosphate (pH 7.0) was added to elute the adsorbed enzyme. The phosphate was exchanged for standard buffer by dialysis. The enzyme was applied to a diethylaminoethyl cellulose column (DEAE) (2.0 × 20 cm), equilibrated with standard buffer, and eluted at 0.2 M-NaCl using a linear NaCl gradient (0 to 0.6 M) in standard buffer. Ten peak tubes (6 ml) were pooled, concentrated with carbowax (Gurr, Romford, Essex), applied to a G-100 Sephadex column (2.0 × 70 cm), and eluted with standard buffer containing 0.2 M NaCl. Ten peak tubes (4.5 ml) were collected from this eluate and concentrated with carbowax. Enzyme preparations were stored at -20 °C.

Enzyme activity was measured with a Unicam SP 1800 spectrophotometer (Shaw & Brodsky, 1968). Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951).

Enzyme homogeneity was assessed by studying the constancy of specific enzyme activity (µmol CoASH liberated/min/mg protein) across the peaks eluted from DEAE cellulose and G-100 Sephadex (except for *recA* (R1 Cm^S/R100-99Tc^R) CAT), and by the appearance of single protein bands on polyacrylamide electrophoresis gels at pH 8.5.

The molecular weights of CATs were determined with G-200 Sephadex (Andrews, 1964) and polyacrylamide gel electrophoresis with sodium dodecyl sulphate (SDS) (Weber & Osborn, 1969).

The stabilities of CATs were determined by heating at 37 °C and withdrawing samples for assay at intervals up to 80 min. As *recA* (R1 Cm^S/R100-99Tc^R) CAT was very unstable when pure, a peak fraction from the DEAE cellulose column was used immediately for this experiment, while a second was collected into a solution of 0.5 mg haemoglobin/ml to determine whether another protein enhanced stability.

Table 1. Purification of R1, 222 and *recA* (R1 Cm^s/R100-99 Tc^R) chloramphenicol acetyl transferases (CATs)

	R1 CAT		222 CAT		<i>recA</i> (R1 Cm ^s /R100 99Tc ^R) CAT	
	Specific activity	Purification	Specific activity	Purification	Specific activity	Purification
Sonicate supernatant	7.9	1.0	4.19	1.0	—	—
Streptomycin precipitate	6.5	0.82	5.19	1.2	0.2	1.0
Ammonium sulphate precipitate	12.2	1.54	8.18	1.95	0.52	2.6
70 °C/10 min	57.7	7.3	21.1	5.0	2.29	11.45
Ammonium sulphate precipitate	143.7	18.1	38.7	9.2	2.44	12.2
C _γ alumina gel	128.0	16.3	93.6	22.3	5.12	25.6
DEAE cellulose pooled peak	114.3	14.5	80.5	19.2	1.6	8.0*
G-100 Sephadex pooled peak	135.0	17.0	119.9	28.6	0.12	0.6*

* Loss in activity during purification.

Antiserum to purified 222 CAT was prepared by injecting a rabbit subcutaneously with protein (4 mg) mixed with an equal volume of Freund's adjuvant; the rabbit was bled after 4 weeks. Reactions between CAT and anti-222 CAT serum were followed by adding anti-222 CAT serum (10 to 80 μ l) to 0.1 ml CAT in a total volume of 0.2 ml. After 2 h at 4 °C the tubes were centrifuged and assayed for CAT. Double diffusion tests (Ouchterlony, 1949) were performed in agar and precipitin bands were observed after 24 h at 4 °C.

RESULTS

Chloramphenicol acetyl transferase was purified from cells containing R1 and 222 and from *recA* (R1 Cm^s/R100-99 Tc^R) (Table 1). When R1 and 222 CATs were eluted from DEAE cellulose and G-100 Sephadex columns the specific activity remained constant through the peaks. *recA* (R1 Cm^s/R100-99 Tc^R) CAT activity was rapidly lost during these steps. All three enzymes gave a single band on polyacrylamide gel electrophoresis; each had the same electrophoretic mobility.

R1 and 222 CAT have a molecular weight of approximately 80000 as determined using G-200 Sephadex (Fig. 1), and subunits of 20000 as determined using SDS electrophoresis (Fig. 2).

K_m determinations. R1 CAT has a *K_m* of 8 to 10 μ M, 222 CAT has a *K_m* of 8 to 10.5 μ M, while *recA* (R1 Cm^s/R100-99 Tc^R) CAT has a *K_m* of 35 to 41 μ M, at least threefold greater than those of CAT enzymes determined by wild-type R factors. Representative results are shown in Fig. 3.

Stabilities of chloramphenicol acetyl transferases at 37 °C. Purified CATs from R1 and 222 were inactivated at 37 °C; pure *recA* (R1 Cm^s/R100-99 Tc^R) CAT was rapidly inactivated, but haemoglobin (0.5%) enhanced its stability (Fig. 4).

Reactions between chloramphenicol acetyl transferase and anti-222 chloramphenicol acetyl transferase serum. Anti-222 CAT serum had an identical neutralizing effect on R1 and 222 CAT (Fig. 5); CAT from *recA* (R1 Cm^s/R100-99 Tc^R) cells was also inactivated, but much

Fig. 1

Fig. 2

Fig. 1. G 200 Sephadex filtration of chloramphenicol acetyl transferase. V_e , elution volume; V_0 , void volume; $\circ$, R1 CAT; Δ , 222 CAT. Standard proteins were obtained from Sigma.

Fig. 2. Electrophoretic mobility of subunits of chloramphenicol acetyl transferase on SDS electrophoresis. Each point represents the average mobility from six experiments. Standard proteins are all from Sigma except for human γ -globulin (Koch-Light Laboratories, Colnbrook, Buckinghamshire). $\circ$, R1 and *recA* (R1 Cm⁸/R100-99Tc^B) CAT; Δ , 222 CAT.

Fig. 3

Fig. 4

Fig. 3. K_m determinations for chloramphenicol. (a) R1 CAT, 5.5 units/ml ($K_m = 10 \mu\text{M}$). (b) 222 CAT, 5.2 units/ml ($K_m = 10.5 \mu\text{M}$). (c) *recA* (R1 Cm⁸/R100-99Tc^B) CAT, 2.4 units/ml ($K_m = 41 \mu\text{M}$). (Units/ml, μmol CoASH liberated/min/ml.)

Fig. 4. Stability of chloramphenicol acetyl transferases (CATs) at 37 °C. $\bullet$, *recA* (R1 Cm⁸/R100-99Tc^B) 0.35 unit/ml purified CAT; $\times$, *recA* (R1 Cm⁸/R100-99Tc^B) 0.15 unit/ml purified CAT + haemoglobin (0.5%); Δ , *recA* (R1 Cm⁸/R100-99Tc^B) 0.38 unit/ml partially purified CAT; $\circ$, R1 2.8 units/ml purified CAT; $\square$, 222 1.6 units/ml purified CAT.

Fig. 5. Inhibition of chloramphenicol acetyl transferase (CAT) activity by anti-222 CAT serum. ○, R1 CAT 2.8 units/ml; □, 222 CAT 2.35 units/ml; ×, *recA* (R1 Cm^s/R100-99Tc^R) CAT 1.7 units/ml; △, *recA* (R1 Cm^s/R100-99Tc^R) CAT 0.16 unit/ml. Anti-222 CAT serum was diluted 1/4.

more antiserum was required to neutralize a similar amount of activity because of the greater amount of protein present. R1 and *recA* (R1 Cm^s/R100-99 Tc^R) CAT showed reactions of identity with 222 CAT in double diffusion tests, as did lysates of R1 Cm^s and R100-99 Tc^R containing cultures. Furthermore, a similar amount of cross-reacting protein was present in lysates of mutants and of cultures containing wild-type R factors. A very low level of CAT activity was detected in mutant culture lysates. Specific enzyme activities (μ mol CoASH liberated/min/mg protein) were as follows: R1 Cm^s, 0.002; R100-99 Tc^R, 0.008; *recA* (R1 Cm^s/R100-99 Tc^R), 0.2; R1, 4; 222, 4.

DISCUSSION

Interallelic complementation occurs in *recA* (R1 Cm^s/R100-99 Tc^R) hetero-R bacteria (Foster & Howe, 1971). They grew slowly on Cm agar, the level of resistance was tenfold lower than that specified by wild-type Cm^R R factors, the specific enzyme activity of the CAT was at least tenfold lower than wild-type, and the presence of both factors in all cells was required for enzyme to be formed.

Wild-type R1 and 222 CAT have a molecular weight of 80000 with subunits of 20000 (cf. *Escherichia coli* R₆/K-10 CAT: Shaw & Brodsky, 1968; Shaw, 1971). The CAT from *recA* (R1 Cm^s/R100-99 Tc^R) differs from that specified by R1 and 222 in having lower affinity for chloramphenicol (as indicated by a threefold higher K_m) and in being very labile when purified. These properties are characteristic of 'complementation enzymes' (Fincham, 1966). Both Cm^s mutants produce a protein which cross-reacts with anti-R₆/K-10 CAT serum. Cm^s mutant cell sonicates have a very low CAT activity, and the additive activity is insufficient to account for that in hetero-R cells.

The finding of interallelic complementation between Cm^s mutants of two R factors is good evidence that CAT is composed of identical subunits. Moreover, it occurs between R factors of different origin. Although this does not necessarily mean that R1 and 222 CATs are identical, they are very similar: they have indistinguishable molecular weights, subunit molecular weights, affinities for chloramphenicol (K_m values: these differ slightly, however, from that of R₆/K-10 CAT; Shaw & Brodsky, 1968; Shaw, 1971), and characteristics of neutralization by antiserum. R factor-mediated chloramphenicol resistance may have arisen by mobilization of a chromosomal gene by a plasmid in *Proteus mirabilis* (Shaw, 1971), and since R factors recombine with one another (see Foster & Howe, 1971), it is possible that R1 and 222 CAT are derived from this source.

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