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Role of Protein Subunits in *Proteus rettgeri* Penicillin G Acylase

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Penicillin G acylase from *Proteus rettgeri* is an 80,000- to 90,000-dalton enzyme composed of two nonidentical subunits. Both subunits were required for enzymatic activity. The 65,000-dalton beta subunit contained a phenylmethylsulfonyl fluoride-sensitive residue required for enzymatic activity, and the 24,500-dalton alpha subunit contained the domain that imparts specificity for the penicillin side chain.

Penicillin G acylase catalyzes the hydrolysis of penicillin G to form 6-aminopenicillanic acid and phenylacetic acid (11). The enzyme is of commercial importance since 6-aminopenicillanic acid is a key intermediate for the synthesis of numerous semisynthetic penicillins (11).

The penicillin G acylase from *Proteus rettgeri* is separated during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) into two subunits (4). Similar findings have been made for the penicillin G acylase from *Escherichia coli* W (4, 8). Kutzbach and Rauenbusch (8) were the first to describe two different-molecular-weight species in crystalline acylase from *E. coli*. They reported that the two polypeptides resulted from incomplete dissociation of the acylase after SDS treatment (8). They concluded from their results that the smaller polypeptide was a monomer of the acylase, whereas the larger polypeptide was the remaining undissociated oligomeric form of the enzyme (8). Recently, Bock et al. (1) showed that the penicillin acylase from *E. coli* was composed of two dissimilar subunits produced from a higher-molecular-weight precursor molecule during secretion (2).

In this communication we report that the subunit structure of the *P. rettgeri* penicillin G acylase is similar to that of that *E. coli* enzyme described by Bock et al. (1) and describe the functional role of these subunits in catalysis. Our study concludes that the *P. rettgeri* acylase is also made up of two nonidentical subunits and that the catalytic site resides in the larger beta subunit, whereas the smaller alpha subunit confers the substrate side chain specificity of the enzyme.

We have previously reported the selection of *P. rettgeri* mutants which acquired the ability to use 6-bromohexanamide (strain Brol) and [1-(4-hydroxy-1-cyclohexenyl)]-acetamide (strain Cycl) as the only nitrogen source (4). The mutants expressed catalytically different penicillin G acylases with new specificity for the penicillin side chain (4). These experimentally evolved acylases were indistinguishable from the wild type enzyme by molecular weight, electrophoretic mobility, or antigenicity (4).

The strain Brol acylase was purified to electrophoretic homogeneity as described previously (4) and treated with 5 M urea. The subunits were separated by the electrophoretic procedure of Davis (5) in polyacrylamide gels containing 5 M urea. The regions of the gels containing each subunit were excised and macerated. The subunits were eluted in buffer (50 mM potassium phosphate, pH 7.5) and analyzed by SDS-PAGE (9). The more basic polypeptide isolated in urea-containing gels corresponded to the 66,000-dalton beta subunit, whereas the more acidic subunit corresponded to the smaller alpha subunit (Fig. 1). Separation of these subunits in either type of denaturing gel, SDS or urea, did not require reducing conditions, which indicated that disulfide bridges were not involved in holding the subunits together. However, the enzyme contained a single disulfide bond which was buried within the structure. Titration of the enzymes with 5,5'-dithiobis-2-nitrobenzoate as described by Pollitt and Zalkin (10) revealed the presence of two sulfhydryl groups per mole of enzyme only after SDS denaturation and dithiothreitol reduction.

The subunits isolated by SDS-PAGE were nonidentical. Limited proteolysis with staphylococcal V8 protease and papain (3) yielded different digestion patterns for each subunit, and the alpha subunit was more resistant to proteolysis than the beta subunit (Fig. 2).

During purification of the acylase by affinity chromatography, it was observed that inactive enzyme, eluted with 6 M urea, could be partially renatured (~50%) by dialysis (4). We

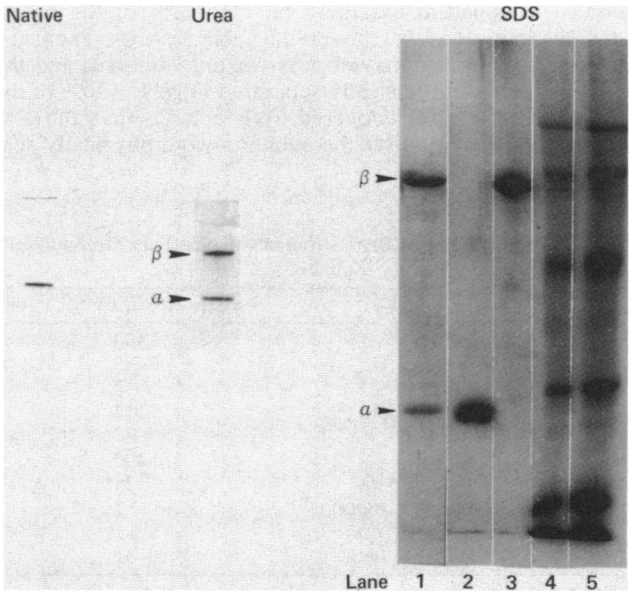

FIG. 1. Resolution of *P. rettgeri* Brol acylase into alpha and beta subunits. A single electrophoretic protein band in native gels resolved into two bands in gels containing urea. In gels containing SDS, the enzyme resolved into a heavy and a light chain (Lane 1). Lanes 2 and 3, Alpha (lane 2) and beta (lane 3) subunits isolated from urea-polyacrylamide gels. Lanes 4 and 5, Protein molecular weight standards (Bethesda Research Laboratories).

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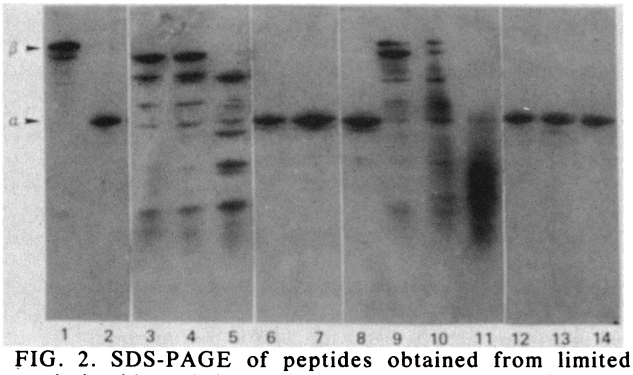

FIG. 2. SDS-PAGE of peptides obtained from limited proteolysis with staphylococcal V8 protease and papain of isolated alpha and beta subunits of the strain Brol acylase. Lanes: 1 and 2, Undigested beta and alpha subunit, respectively; 3 through 5, beta subunit plus 0.05, 0.1, and 0.5 μg of V8 protease, respectively; 6 through 8, alpha subunit plus 0.05, 0.1, and 0.5 μg of V8 protease, respectively; 9 through 11, beta subunit plus 0.05, 0.1, and 0.5 μg of papain, respectively; 12 through 14, alpha subunit with 0.05, 0.1, and 0.5 μg of papain, respectively.

took advantage of this property to design subunit mixing experiments. The acylase subunits were isolated from urea-polyacrylamide gels as described above. To allow the subunits to refold, they were dialyzed overnight at room temperature against a sterile solution of 50 mM potassium phosphate buffer, pH 7.5.

Neither the alpha nor beta subunit regained enzymatic activity when dialyzed separately, and no activity was recovered when these subunits were mixed after dialysis. In contrast, when the subunits were mixed in the presence of urea and allowed to refold together during dialysis, activity was regained (Table 1, experiment 1). These results indicate that both subunits were required for enzymatic activity.

When the subunits were physically separated in gels and allowed to renature together, ca. 6 to 12% of the native enzyme activity was recovered (Table 1, experiment 1). However, when the enzyme was treated with urea and the subunits were not physically separated in gels, ~50% of the native activity was recovered. We believe that the low recovery of activity after the subunits were physically separated was partly because the protein was not completely recovered from the gels during elution and partly due to proteolytic degradation of the acylase. The enzyme preparations had either latent endogenous proteolytic activity or a minor contaminating protease which was only evident in the presence of urea. If a proteolytic contaminant was present in the enzyme preparation, it must have had the same electrophoretic mobility as the acylase. Active acylase bands eluted from polyacrylamide gels showed a similar lack of stability after treatment with urea. Furthermore, the beta subunit was much more stable in urea when the alpha subunit was present. In this respect the acylase seemed to behave very much like rat kidney gamma-glutamyl transpeptidase (6). Rat kidney gamma-glutamyl transpeptidase is also made up of two unequal subunits, and their dissociation in urea unmasks an endogenous protease which preferentially degrades the larger subunit (6).

The Cycl and Brol strain enzymes apparently contained an essential serine residue, as they were completely inactivated by phenylmethylsulfonyl fluoride (PMSF). For these inactivation studies, stock PMSF solutions (10 mM) were freshly prepared in isopropyl alcohol, and the enzymes were incubated with the inhibitor for 20 min at various molar ratios. One mole of enzyme was completely inactivated by one mole of PMSF. Isopropyl alcohol alone had no effect on enzyme activity.

To determine which subunit contained the PMSF-sensitive residue, subunits from native and PMSF-inactivated enzymes (1 mol of PMSF per mol of enzyme) were isolated, and various combinations of alpha and beta subunits were mixed and renatured by dialysis. The resulting activities from the various mixtures for the Brol strain acylase are shown in Table 1, experiment 2. Partial enzymatic activity was restored only when the untreated beta subunit was the source of the beta polypeptide. In either mixture, when the beta subunit was derived from PMSF-treated acylase no enzymatic activity was detected. These results strongly suggested that the beta subunit contained a PMSF-sensitive residue (serine) essential for catalysis. They also imply that the alpha subunit played an important role in catalysis since the beta subunit alone had no enzymatic activity. The Cycl strain acylase gave the same results (data not shown).

The Brol and Cycl strain acylases are catalytically different (4). The Brol acylase is ~53 times more specific (V_{max}/K_m) for the *N*-(3-carboxy-4-nitrophenyl) (NCN)-6-bromohexanamide model substrate than the Cycl enzyme (4). Its V_{max} was 12 times greater for this substrate than the

TABLE 1. Recovery of Brol strain acylase activity after subunit mixing[a]

Expt no.[b]	Subunit(s)	Acylase activity (% of control)
1	Control[c]	100
	Beta	1
	Alpha	0
	Alpha + beta	12
2	Control	100
	Alpha + beta	25
	(Alpha) + (beta)	1
	Alpha + (beta)	1
	(Alpha) + beta	31

[a] Acylase activity was measured by the hydrolysis of NCN-phenylacetamide as described previously (4).

[b] In experiment 1, the alpha and beta subunits were separated and allowed to renature alone or mixed together. In experiment 2, subunits shown in parentheses were isolated from PMSF-treated enzyme and the other subunits were isolated from untreated enzyme.

[c] In the controls, the enzyme was treated with urea but the subunits were not physically separated. The recovery of activity was 50% of the native enzyme activity.

TABLE 2. Subunit mixing for hybrid enzymes

Subunits[a]		Acylase recovered[b] (U)		Ratio (NCNPA/NCNBR)
Alpha	Beta	NCNPA	NCNBR	
Brol	Brol	0.029	2.4×10^{-3}	12
Cycl	Cycl	0.035	6.7×10^{-5}	522
Cycl	Brol	0.052	6.7×10^{-5}	776
Brol	Cycl	0.032	5.1×10^{-3}	6

[a] Brol and Cycl strain acylases (50 mg) (7) were resolved in gels containing urea. The alpha and beta subunits were excised and mixed in the combinations indicated and then allowed to renature overnight during dialysis.

[b] Acylase recovered from subunit mixtures after renaturation. NCNPA, NCN-phenylacetamide; NCNBR, NCN-6-bromohexanamide. The substrate concentration used to assay the enzymes was 2.4 mM. The apparent K_m of the Brol and Cycl enzymes was 0.05 and 0.02 mM for NCN-phenylacetamide, respectively, and 0.3 and 0.5 mM for NCN-6-bromohexanamide, respectively (4).

Cycl acylase. However, both enzymes have a similar V_{max} for NCN-phenylacetamide (4). To determine the role of the subunits in defining enzyme specificity, the alpha and beta subunits from the Brol and Cycl acylases were isolated from urea-polyacrylamide gels and mixed in several combinations. The reconstituted parent and hybrid enzymes were then assayed for hydrolysis of the NCN-phenylacetamide and NCN-6-bromohexanamide acylase model substrates (Table 2). The subunits from both enzymes reassociated with equal efficiency. Each renatured parental and hybrid enzyme had similar activity for the NCN-phenylacetamide substrate. The hybrid enzyme with the alpha subunit from the Brol strain acylase had substantial activity against the NCN-6-bromohexanamide model substrate; however, the hybrid enzyme with the alpha subunit from strain Cycl had very low levels of activity for this side chain. These results indicate that the role of the alpha subunit in catalysis is to provide the enzyme with its side chain specificity.

In summary, the results presented in this communication are consistent with the conclusion that the *P. rettgeri* acylases are made up of two nonidentical subunits, alpha and beta, which have distinct and independent functions. Specifically, the beta subunit contributes a serine residue essential for catalysis, and the alpha subunit provides substrate specificity to the enzyme. Together the subunits define the acylase active site.

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