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High-Molecular-Weight Penicillin-Binding Proteins from Membranes of Bacilli

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Mixtures of high-molecular-weight, cephalosporin-sensitive penicillin-binding proteins (PBPs) can be purified from *Bacillus subtilis* membranes by cephalosporin affinity chromatography (G. Kleppe and J. L. Strominger, J. Biol. Chem. **254**:4856–4862, 1979). By appropriate modification of this technique, *B. subtilis* PBP 1 was purified to homogeneity, and a mixture of *Bacillus stearothermophilus* PBPs 1, 2, and 4 was isolated. [^{14}C]penicillin-PBP complexes of high-molecular-weight PBPs purified from membranes of these two bacilli, after denaturation, were found to have chemical reactivities typical of the penicilloyl-serine derivative formed by D-alanine carboxypeptidase from *B. stearothermophilus*. Although enzymatic activity catalyzed by these and several other high-molecular-weight PBPs from gram-positive organisms has not been detected with cell wall-related substrates, a slow, enzymatic acylation of *B. subtilis* PBPs 1, 2ab, and 4 by [^{14}C]-diacetyl-L-lysyl-D-alanyl-D-lactate was demonstrated. Further study is necessary to clarify the physiological relevance of the slow acylation by this analog of a natural cell wall biosynthetic intermediate.

Several distinct proteins which bind penicillin and related β-lactam antibiotics covalently have been detected in bacterial membranes (3, 15, 16, 23, 28). Genetic and biochemical studies of these penicillin-binding proteins (PBPs) evidence their importance as essential enzymes in the biosynthesis of cell wall peptidoglycan. PBPs have been purified recently from several organisms by covalent penicillin affinity chromatography (2) and can be shown to comprise two categories. (i) High-molecular-weight PBPs which often account for only 10 to 30% of the total penicillin-binding activity of an organism are sensitive to low concentrations of both penicillins and the related cephalosporin antibiotics. Recent studies suggest that these PBPs include penicillin-sensitive transpeptidases essential for bacterial growth and division (14, 19). (ii) Low-molecular-weight PBPs usually consist of a single major PBP which is sensitive to low concentrations of penicillins but insensitive to high concentrations of cephalosporins. These low-molecular-weight PBPs catalyze a penicillin-sensitive D-alanine carboxypeptidase (CPase) reaction in vitro and, under certain conditions, a related transpeptidase reaction as well (3, 7, 23). In contrast to the essential nature of the high-molecular-weight PBPs, the bulk of CPase activity seems to be unessential for cell survival in several cases (1, 11).

Mixtures of high-molecular-weight PBPs have been purified from *Bacillus subtilis* by cephalosporin affinity chromatography (8), and some of their biochemical properties have been studied. In particular, the following have been shown. (i) *B. subtilis* PBPs 1, 2b, 4, and 5 each have distinct [^{14}C]penicilloyl-peptides (8). (ii) Antibody raised to PBP 5 (the CPase) does not cross-react with any of the high-molecular-weight PBPs (4). (iii) Mixtures of PBPs 1, 2ab, and 4 catalyze release of the covalently bound [^{14}C]penicilloyl moiety to yield [^{14}C]phenylacetylglycine as the principal fragmentation product (21), a reaction also characteristic of several CPases. (iv) Mixtures of high-molecular-weight PBPs do not catalyze detectable transpeptidase or D-alanine carboxypeptidase reactions in vitro with either conventional cell wall-related substrates or a linear, un-cross-linked peptidoglycan isolated from penicillin-treated *B. subtilis* (8, 25). The apparent absence of such activities has led us to further examine in this study the question as to whether the high-molecular-weight PBPs are biochemically similar to the low-molecular-weight PBPs as reflected by (i) the chemical nature of the penicilloyl-PBP linkage and (ii) the interactions of high-molecular-weight PBPs with several synthetic cell wall-related compounds including diacetyl-L-lysyl-D-

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alanyl-D-lactate, an excellent substrate for several low-molecular-weight PBPs (13).

MATERIALS AND METHODS

[^{14}C]penicillin G (51 to 56 mCi/mmol) was obtained from Amersham Corp., and [^{14}C]diacetyl-L-lysyl-D-alanyl-D-lactate (118 mCi/mmol) was synthesized as described previously (13). Membranes were prepared from *B. subtilis* strain Porton and *Bacillus stearothermophilus* ATCC 15952 as described previously for *B. subtilis* (22). Materials used for β-lactam affinity chromatography and for sodium dodecyl sulfate gel electrophoresis were described previously (20). 6-Aminopenicillanic acid–Sepharose (penicillin affinity resin) and 7-aminocephalosporanic acid–Sepharose (cephalosporin affinity resin) were prepared as described previously (8, 20) except that the cephalosporin resin was treated with 1 M glycine-NaOH, pH 9.0 (15 min at 25°C), before a final wash with 0.02 M KPO_4 (pH 7.0)–0.5 M NaCl.

Sodium borohydride, Triton X-100, and penicillin G were obtained from Sigma Chemical Co., and mixtures of protein molecular weight standards were obtained from Bio-Rad Laboratories. Unlabeled peptide substrates were those described previously (8).

A mixture of *B. subtilis* PBPs 1, 2ab, and 4 was isolated by cephalosporin affinity chromatography (8). Dialysis of the mixture eluted from the cephalosporin affinity resin against 10 mM Tris-chloride (pH 8.6)–0.1% Triton X-100 at 4°C resulted in the selective precipitation of PBPs 2ab and 4, with PBP 5 and the majority of PBP 1 remaining soluble. Three higher-molecular-weight proteins found in these PBP mixtures (5, 8) were of M_r = 155,000, 147,000, and 138,000, suggesting that they might be dimers of PBP 2a (M_r = 77,000), PBP 2b (M_r = 75,000), and PBP 4 (M_r = 68,000), respectively. *B. subtilis* PBP 1 was purified by a modification of the affinity chromatography technique as described in the legend to Fig. 1. A mixture of high-molecular-weight PBPs was purified from *B. stearothermophilus* membranes as described for *B. subtilis* (8), except that the PBPs were concentrated on sulfopropyl-Sephadex equilibrated in 10 mM sodium acetate (pH 4.0)–0.1% Triton X-100 after dialysis against this same buffer.

[^{14}C]penicillin G (10 to 40 μg/ml) was bound covalently to mixtures of PBPs (0.1 to 3 mg of protein per ml; pH ranging from 5 to 9.5) by incubation for 10 min at 25°C. Penicillin binding was assayed by fluorography after discontinuous sodium dodecyl sulfate gel electrophoresis (7.5% gels) essentially as described previously (5). Rapid quantitation of covalently bound [^{14}C]penicillin G- or [^{14}C]diacetyl-L-lysyl-D-alanyl-D-lactate-derived label was effected by a filter binding assay (20). Radioactivity was determined by liquid scintillation counting in a toluene-based fluid at an efficiency of 70%. Hydrolysis of [^{14}C]diacetyl-L-lysyl-D-alanyl-D-lactate to [^{14}C]diacetyl-L-lysyl-D-alanine was determined by high-voltage paper electrophoresis (13).

RESULTS

Purification of PBPs. Previous studies have shown that the *B. subtilis* [^{14}C]penicilloyl-PBP 1 complex catalyzes an enzymatic release of its bound penicilloyl moiety with a half-life of approximately 10 min at 37°C (21). By contrast, half-lives for the other *B. subtilis* penicilloyl-PBP complexes are significantly longer (PBP 2, ~300 min; PBP 4 ~60 min; and PBP 5, ~120 min at 37°C) (21). This rapid release of bound penicillin catalyzed by PBP 1 suggested the purification scheme utilized in Fig. 1. Penicillin G was prebound to *B. subtilis* membranes, and a Triton X-100 extract was then incubated with an affinity resin containing either a cephalosporin or a penicillin moiety. Only PBP 1 releases the prebound penicilloyl moiety sufficiently rapidly to allow it to subsequently bind to the β-lactam affinity resins (Fig. 1, lanes 1 to 4), the other PBPs flowing through without binding. The PBP 5 trapped on the penicillin affinity column (lane 2) reflects penicillin release from a small fraction of the large amount of this protein initially present in the membranes ([^{14}C]penicillin-binding activity ratio of PBP 5 to PBP 1 ~ 10 [8]). The low affinity of PBP 5 (the CPase) for the cephalosporin affinity column (8) facilitates the purification of *B. subtilis* PBP 1 (Fig. 1, lanes 1, 3, and 4). Purified PBP 1 was shown to form a covalent [^{14}C]penicilloyl-PBP complex which, after isolation by gel filtration through Sephadex G-50, contained 1.15 mol of [^{14}C]penicillin bound per mol of protein (determined as for the *B. stearothermophilus* CPase [20], assuming a molecular weight of 105,000 for PBP 1).

A mixture of high-molecular-weight, cephalosporin-sensitive PBPs (PBPs 1, 2, and 4) was readily purified from Triton X-100-solubilized *B. stearothermophilus* membranes by covalent cephalosporin affinity chromatography, as described for *B. subtilis* (8). The results obtained (Fig. 1, lanes 5 and 6) indicated apparent molecular weights for *B. stearothermophilus* PBPs, 1, 2, and 4 which are quite similar to those of *B. subtilis* PBPs 1, 2ab, and 4, respectively. *B. stearothermophilus* PBP 2 was seen to consist of several bands upon gel electrophoresis. In contrast to *B. subtilis* PBP 1, neither purified *B. stearothermophilus* PBP 1 nor the other high-molecular-weight PBPs of *B. stearothermophilus* catalyzed a rapid release of the bound [^{14}C]penicilloyl moiety (half-life, >1.5 h at 37°C). In addition, each of the high-molecular-weight *B. stearothermophilus* PBPs present in the mixture precipitated upon dialysis to low ionic strength (10 mM sodium acetate [pH 4]–0.1% Triton X-100).

Nature of penicilloyl-PBP linkage. Several studies of the interactions of high-molecular-weight PBPs with β-lactams suggest that

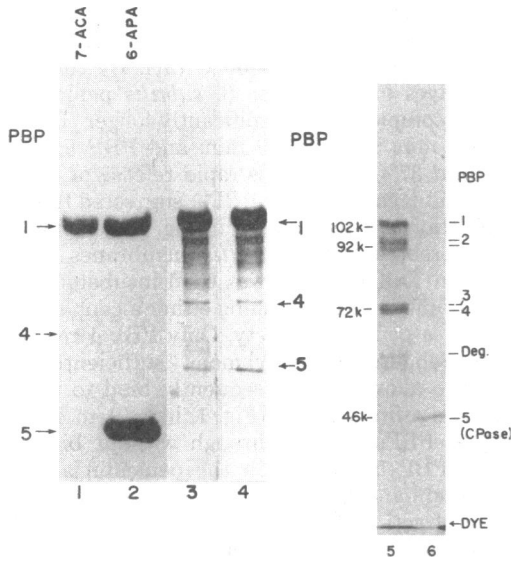

FIG. 1. *Sodium dodecyl sulfate gels of purified PBPs. Lanes 1 to 4 show the purification of B. subtilis PBP 1 from penicillin G-pretreated membranes by cephalosporin affinity chromatography. Membranes were incubated with penicillin G (100 μg/ml for 10 min at 25°C), and excess antibiotic was removed by two washings. Membranes were then solubilized with 2% Triton X-100 and incubated with either 7-aminocephalosporanic acid (7-ACA)–Sepharose (lane 1) or 6-aminopenicillanic acid (6-APA)–Sepharose (lane 2). Shown is a sample of the hydroxylamine eluent from each Sepharose resin as analyzed by sodium dodecyl sulfate gel electrophoresis and fluorography. PBP 1 releases its prebound penicilloyl moiety rapidly (21) and can therefore be bound by either affinity resin. The amount of PBP 5 trapped on the penicillin affinity resin is approximately 10% as much as that bound in the absence of penicillin G pretreatment. Small amounts of PBP 4 were also trapped by the cephalosporin affinity resin. Lanes 3 and 4 show the same experiment as lane 1, except that several proteolytic fragments of PBP 1 are present between PBPs 1 and 4. PBPs are revealed by Coomassie blue staining (lane 3) and by fluorography (lane 4). Lane 5 is a Coomassie blue-stained gel showing PBPs 1, 2, and 4 purified from B. stearothermophilus membranes by use of a cephalosporin affinity column (8). Lane 6 shows PBP 5 (the D-alanine carboxypeptidase, CPase) purified from the effluent of the column by penicillin affinity chromatography. PBP 3 (M_r ~ 74,000) was not recovered by these methods. Apparent molecular weights are as indicated on the left. Deg., Bands arising from proteolytic degradation of the PBPs upon storage.*

these proteins bind and release β-lactam antibiotics in a manner which is equivalent to the processing of β-lactams by CPases (21, 23). Thus, the penicilloyl-PBP complexes formed with high-molecular-weight PBPs might be not only enzymatically equivalent but also chemically equivalent to those formed with the CPases. To test this possibility, penicilloyl-PBP complexes were formed by incubating [^{14}C]penicillin G with *B. stearothermophilus* CPase, *B. stearothermophilus* PBPs 1, 2, and 4, and *B. subtilis* PBPs 1, 2, and 4 and then were denatured by heat inactivation. The extent of chemical release of the bound [^{14}C]penicilloyl moiety upon treating the mixtures of PBPs under a variety of conditions was then evaluated. The results (Table 1) suggest that penicillin is bound to the high-molecular-weight PBPs in a linkage which is sensitive to base hydrolysis and somewhat sensitive to mild reduction by sodium borohydride. Since the penicilloyl-CPase complex, known to contain a penicilloyl-serine ester linkage (22, 26, 27), exhibits the same chemical stability (Table 1), it is likely that penicillin is also bound as a serine (or threonine) ester by the high-molecular-weight PBPs (9).

Interaction of high-molecular-weight PBPs with cell wall-related substrates. Attempts to demonstrate either transpeptidase or D-alanine carboxypeptidase activity by high-molecular-weight PBPs from *B. subtilis* or from

TABLE 1. *Chemical stability of penicilloyl-PBP linkage*[a]

Treatment	% [^{14}C]penicillin[b] bound to:		
	B. subtilis PBPs 1, 2, and 4	*B. stearothermophilus* PBPs 1, 2, and 4	*B. stearothermophilus* CPase
0.5 M NH_2OH, pH 7.0 (native)[d]	68.1[c]	50.6[c]	2.0
0.5 M NH_2OH, pH 7.0	99.3	98.7	96.0
0.5 M Tris-chloride, pH 9.3	89.9	95.1	97.2
0.5 M Tris-chloride, pH 9.3, 5 mM $NaBH_4$	84.4	79.5	99.4
0.5 M Tris-chloride, pH 9.3, 25 mM $NaBH_4$	62.0	74.1	79.5
0.5 M Tris-chloride, pH 9.3, 125 mM $NaBH_4$	38.3	41.1	55.7
0.25 M NaOH	5.2	2.9	4.4

[a] [^{14}C]penicillin G was bound to the PBPs (0.3 to 0.6 nmol/ml in 0.1 M Tris-chloride [pH 7.5]–0.7% Triton X-100) for 10 min at 37°C, and a 100-fold excess of cold penicillin G was then added, after which the samples were boiled for 2 min. Samples either were untreated (control) or were diluted 10-fold into the buffers indicated in column 1 and incubated for 30 min at 37°C, after which residual [^{14}C]penicillin-PBP complexes were quantitated by filter binding and liquid scintillation counting after precipitation with 10% trichloroacetic acid.

[b] Control values, representing 100%, were 2,600, 6,300, and 4,500 cpm for *B. subtilis* PBPs 1, 2, and 4, *B. stearothermophilus* PBPs 1, 2, and 4, and *B. stearothermophilus* CPase, respectively.

[c] Enzymatic release was more complete upon longer incubation.

[d] This sample was not boiled.

other gram-positive bacteria have been unsuccessful (6, 8, 10, 25). Efforts to detect inhibition of [^{14}C]penicillin G binding to *B. subtilis* PBPs 1, 2, and 4 (assayed by sodium dodecyl sulfate gel electrophoresis and fluorography and measured under conditions in which the penicillin is not saturating) in the presence of either 40 mM diacetyl-L-lysine-D-alanyl-D-lactate, 20 mM diacetyl-L-lysyl-D-alanyl-D-alanine, or 20 mM *N*-acetyl-D-alanyl-D-alanine (either at pH 4 or 8.5) were also unsuccessful.

Incubation of *B. subtilis* PBPs 1, 2, and 4 with [^{14}C]diacetyl-L-lysyl-D-alanyl-D-lactate for extended times resulted in a slow acylation of these PBPs (Fig. 2). Acylation of each of these high-molecular-weight PBPs was blocked by pretreatment with the cephalosporin cephalothin or by heat or detergent denaturation and was likewise not observed with ovalbumin as a control protein (Fig. 3). Thus, the reaction may result from specific interactions between PBPs and substrate. Acylation occurred both at pH 9.5 and at a two- to threefold-slower rate (data not shown) at pH 4.0. (Residual CPase activity due to the trace amount of PBP 5 present precluded studies of the acylation in the range pH 5 to 7.5.)

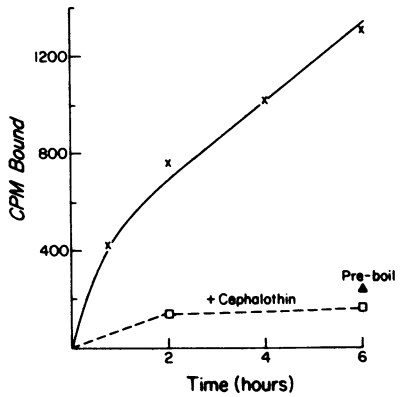

FIG. 2. *Accumulation of acyl-enzyme by mixtures of B. subtilis PBPs 1, 2ab, and 4. PBPs (0.6 nmol of [^{14}C]penicillin G-binding activity suspended in 10 μl of 0.1 M sodium borate [pH 9.5]–0.1% Triton X-100) were incubated with [^{14}C]diacetyl-L-lysyl-D-alanyl-D-lactate (1.2 μCi, ~10 nmol) in a final volume of 15 μl for the indicated times at 25°C. Acylation was halted by the addition of 0.5 ml of cold 10% trichloroacetic acid, after which [^{14}C]acyl-protein complexes were quantitated by filter binding. The decrease in the rate of acylation with time is possibly due to partial denaturation and to the conversion of the ^{14}C-labeled substrate to [^{14}C]diacetyl-L-lysyl-D-alanine by the trace amounts of B. subtilis PBP 5 (CPase) present in the incubation mixture (35% hydrolysis of the substrate by 6 h). A total of 1,300 cpm bound at 6 h corresponds to approximately 1.3% complex formation (relative to [^{14}C]penicillin G binding). Control samples were heat inactivated (60 s at 100°C) or treated with cephalothin (135 μg/ml; approximately 5 nmol) before addition of the ^{14}C-labeled substrate, as indicated.*

DISCUSSION

Biochemical studies of high-molecular-weight PBPs have been undertaken to help elucidate the functions of these membrane proteins in vivo and the precise mechanisms by which β-lactam antibiotics inhibit their catalytic activities, leading to cell death. To this end, several recent studies have focused on the purification of high-molecular-weight PBPs, with the hope of determining the enzymatic reactions catalyzed (6, 8, 19). In this study PBP 1 has been purified from *B. subtilis* membranes, taking advantage of the

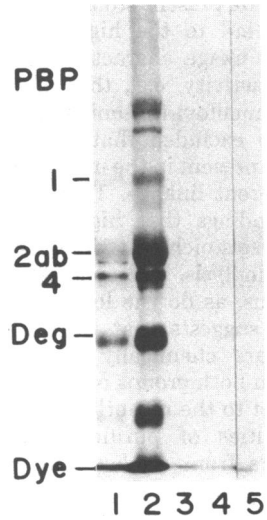

FIG. 3. *Gel electrophoresis of a mixture of B. subtilis PBPs 1, 2ab, and 4 acylated by [^{14}C]diacetyl-L-lysyl-D-alanyl-D-lactate. PBPs (~15 μg suspended in 5 μl of 0.1 M sodium borate [pH 9.5]–0.1% Triton X-100) were incubated with [^{14}C]diacetyl-L-lysyl-D-alanyl-D-lactate (5 μl at 118 mCi/mmol; final concentration ~ 1 mM) for 30 min (lane 1) or 150 min (lane 2) at 37°C. Samples were then acetone precipitated and analyzed by sodium dodecyl sulfate gel electrophoresis and fluorography (3-week exposure). In control experiments PBPs were heat denatured (60 s at 100°C, lane 3), incubated with sodium dodecyl sulfate (final concentration, 1%; lane 4), or treated with cephalothin (100 μg/ml for 10 min at 25°C, approximately a 10-fold molar excess over PBPs; lane 5) before addition of the ^{14}C-labeled substrate. In cases in which [^{14}C]penicillin G was used or in which [^{14}C]diacetyl-L-lysyl-D-alanyl-D-lactate was used at pH 4 instead of pH 9.5, the pattern of labeling differed in that the bands marked "Deg" and the lower-molecular-weight bands present at the dye front (most of which arise from proteolytic degradation of the PBPs upon storage) were labeled much less intensely.*

rapid release of a bound penicilloyl moiety catalyzed by this enzyme (21).

A mixture of *B. stearothermophilus* PBPs 1, 2, and 4 could also be purified, with the major PBP (PBP 5, the D-alanine carboxypeptidase) present in only trace amounts, by application of the technique of cephalosporin affinity chromatography (8).

[^{14}C]penicillin G forms a stoichiometric, covalent complex with *B. subtilis* PBP 5 (24, 27) which, after denaturation, is stable to NH_2OH and somewhat sensitive to borohydride reduction at pH 9, but is readily cleaved under alkaline conditions (pH 12) (9). Amino acid sequence analysis of [^{14}C]penicilloyl-peptides derived from both *B. subtilis* and *B. stearothermophilus* PBP 5 established this alkali-labile linkage as a penicilloyl ester of serine 36 (22, 26, 27). Results obtained in the present study indicate that penicillin is bound to the high-molecular-weight PBPs in a linkage characterized by a similar chemical reactivity, e.g., that of a penicilloyl-serine or penicilloyl-threonine ester. It cannot, however, be excluded that one of the minor components present in the mixture of PBPs may form a different linkage. This result, together with the findings that high-molecular-weight PBPs catalyze stoichiometric penicillin binding, hydroxylaminolysis, and enzymatic fragmentation reactions, as do the low-molecular-weight PBPs (23), suggests that the penicilloyl-PBP complexes are chemically and enzymatically equivalent in both groups of PBPs.

In contrast to the recently demonstrated catalytic activities of purified, high-molecular-weight PBPs from *Escherichia coli* (12, 19), attempts to demonstrate transpeptidase or D-alanine carboxypeptidase reactions catalyzed by high-molecular-weight PBPs from several gram-positive organisms have thus far been unsuccessful. A slow acylation of *B. subtilis* PBPs 1, 2, and 4 could, however, be detected in the present study with the synthetic D-alanine carboxypeptidase substrate [^{14}C]diacetyl-L-lysyl-D-alanyl-D-lactate. That acylation was effectively blocked by the β-lactam cephalothin or by heat or detergent denaturation argues for a specific interaction between β-lactam and substrate. Under similar incubation conditions, *E. coli* PBP 1A, but not *E. coli* PBP 1Bs, is also acylated (H. Amanuma and J. L. Strominger, unpublished data). Possible reasons for the lack of more readily demonstrable in vitro activities catalyzed by these and other high-molecular-weight PBPs from gram-positive organisms might include the following. (i) Despite their high efficiency as substrates for reactions catalyzed by low-molecular-weight PBPs, the synthetic peptides and other cell wall-related compounds utilized in vitro are inappropriate substrates for the as yet undetermined reactions catalyzed by high-molecular-weight PBPs in vivo. In addition, an amino acceptor might be required even for the acylation reaction; e.g., it might be needed to induce a conformational change in the enzyme. (ii) High-molecular-weight PBPs might require an appropriate lipid environment for activity in vitro, a possibility which has not been investigated sufficiently. (iii) Although purification of high-molecular-weight PBPs does not, in general, lead to loss of penicillin-binding or penicilloyl fragmentation activity, purification may effect partial denaturation or, alternatively, remove an effector or cofactor required for efficient processing of this and other cell wall-related compounds. (iv) Transpeptidation is the result of a complicated process requiring the coupled and possibly concerted activity of several membrane enzyme systems. Thus, at least with certain PBPs, one might not be able to demonstrate catalytic activity with synthetic substrate and the purified proteins unless they are reconstituted together with other peptidoglycan biosynthetic enzymes. Recent advances in the resolution and reconstitution of several such enzymes (17, 18) may prove useful in this regard.

ACKNOWLEDGMENT

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