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Structural Features of Polysaccharides That Induce Intra-Abdominal Abscesses

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The capsular polysaccharide complex from *Bacteroides fragilis* promotes the formation of intra-abdominal abscesses—a pathologic host response to infecting microorganisms. This complex consists of two distinct polysaccharides, each with repeating units that have positively charged amino groups and negatively charged carboxyl or phosphate groups. Analysis of these polysaccharides as well as other charged carbohydrates before and after chemical modification revealed that these oppositely charged groups are required for the induction of intra-abdominal abscesses in a rat model.

The mechanisms by which bacterial pathogens induce specific disease processes are often poorly understood. Intra-abdominal abscess formation caused by *Bacteroides fragilis* is an example of a pathologic host response to infection. In this tissue reaction, a fibrous capsule localizes invading bacteria and presumably protects the host from disseminated infection. These abscesses cause substantial morbidity and mortality, are difficult to treat with antimicrobial therapy, and usually require surgical intervention (*1*).

Although *B. fragilis* makes up less than 0.5% of the normal colonic microflora, it is the predominate obligate anaerobe isolated from human infections such as intra-abdominal sepsis and bacteremia (*2*, *3*). In rodent models, intraperitoneal administration of *B. fragilis* or its capsular polysaccharide complex (CPC) promotes the formation of intra-abdominal abscesses (*4*). Abscesses formed in response to the CPC are bacteriologically sterile yet histologically identical to abscesses formed in response to intact bacteria. Although most bacterial polysaccharides are considered to be T cell–independent antigens that elicit humoral responses, the CPC promotes abscess formation and confers immunity to abscess induction in a T cell–dependent manner (*5*).

The CPC of *B. fragilis* 9343 exhibits unusual chemical and immunochemical characteristics (*6*, *7*). Recently, we have shown that this complex consists of two distinct high molecular weight polysaccharides, termed A and B (*8*). Each is composed of multiple repeating units of oligosaccharides that have uncommon constituent sugars with free amino, carboxyl, and phosphate groups (*9*) (Fig. 1). Polysaccharide A has a tetrasaccharide repeating unit with a balanced positively charged amino group and negatively charged carboxyl group (Fig. 1A). Polysaccharide B has a hexasaccharide repeating unit, including an unusual 2-aminoethylphosphonate substituent containing a free amino group and a negatively charged phosphate group. The galacturonic acid residue contains an additional negatively charged carboxyl group (Fig. 1B). Ionic interactions between the two saccharide chains tightly link polysaccharides A and B into a high molecular weight complex (*10*). Further study has shown that all strains of *B. fragilis* examined thus far also have a complex of at least two different polysaccharides that are antigenically diverse while some strains show cross-reactivity with the 9343 CPC (*11*).

We hypothesized that the unusual structural features of the CPC are critical to abscess formation, and therefore tested polysaccharide A and B and the CPC for the ability to induce abscesses in a rat model of intra-abdominal sepsis (*12*). Rats were injected intraperitoneally with each polymer, and the dose required to induce abscesses in 50% of the animals (AD_{50}) was determined. In this assay, polysaccharide A was an order of magnitude more active (AD_{50} = 0.67 μg) than polysaccharide B (AD_{50} = 25 μg) or the CPC (AD_{50} = 22 μg) (Table 1).

Table 1. Abscess induction by *B. fragilis* polysaccharides. Rats were administered 10-fold dilutions of each polysaccharide mixed 1:1 with a sterile cecal contents adjuvant (*4*, *12*) and examined 6 days later for the formation of intra-abdominal abscesses. Rats receiving saline (no polysaccharide) and adjuvant did not form abscesses in these experiments. Data were accumulated from two separate experiments. Experiment 1: *B. fragilis* component polysaccharides and the CPC were tested. The AD_{50} values were calculated by the method of Reed and Muench (*25*). Experiment 2: Chemically modified versions of polysaccharide A were tested in the rat model. Chemical modifications of polysaccharide A are detailed in Fig. 1A. The AD_{50} and *P* values were calculated with the use of a mathematical model based on logistic regression analysis (*26*). ND, not done.

Polysaccharide	Fraction of rats with abscesses at a dose of					AD_{50} (μg)	*P*
	200 μg	20 μg	2 μg	0.2 μg	0.02 μg		
			Experiment 1				
A (native)	31/38	18/25	21/38	7/18	2/19	0.67	—
B	23/29	14/30	5/28	ND	ND	25	—
CPC	23/28	10/19	6/20	ND	ND	22	—
			Experiment 2				
A (native)	16/20	14/20	10/19	ND	ND	1.3	—
A (reduced)	5/20	2/19	2/19	ND	ND	>200	<0.0005*
A (*N*-acetylated)	7/20	3/19	1/17	ND	ND	>200	<0.0005*
A (deaminated)	7/20	6/18	3/19	ND	ND	>200	<0.0005*

*As compared with polysaccharide A (native).

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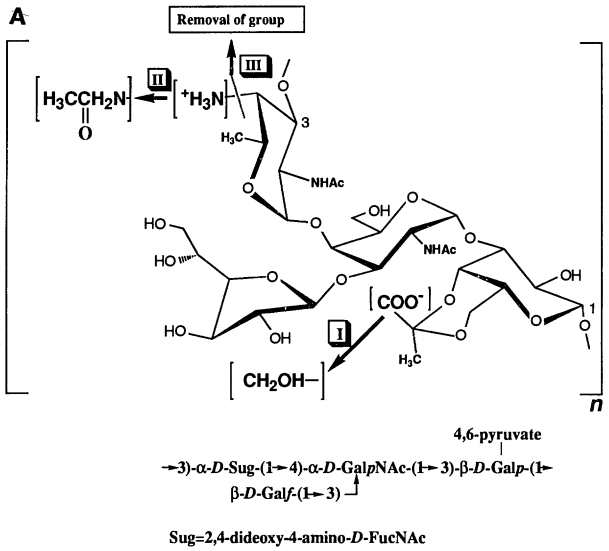
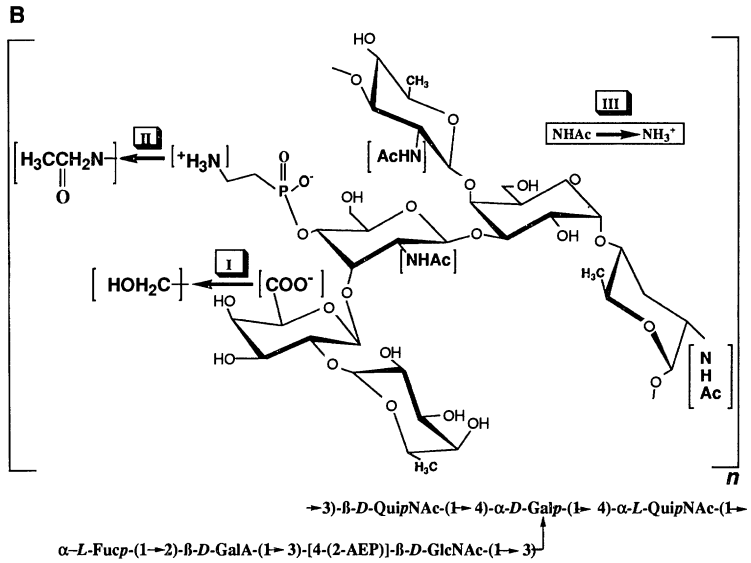
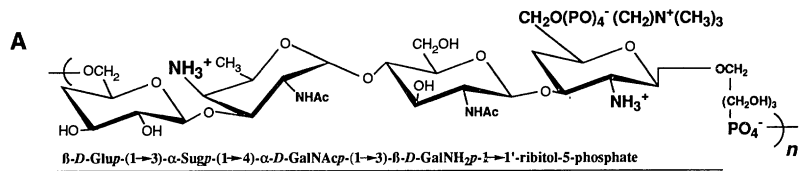
We then tested whether specific structural aspects of the *B. fragilis* polysaccharides modulate abscess induction. Polysaccharides A and B were chemically modified to eliminate the positively or negatively charged groups on each repeating unit (Fig. 1). Chemical modifications were verified by nuclear magnetic resonance (NMR) spectroscopy (9). Modifications to polysaccharide A included (i) carbodiimide reduction of the negatively charged carboxyl group associated with the pyruvate substituent to a neutral hydroxymethyl group (*13*), (ii) *N*-acetylation of the free amino group (9) on the trideoxyfucosamine, and (iii) removal of the same free amino group by nitrous acid deamination (9). Removal of acetyl groups was not attempted on polysaccharide A, as the reaction conditions would depolymerize this saccharide.

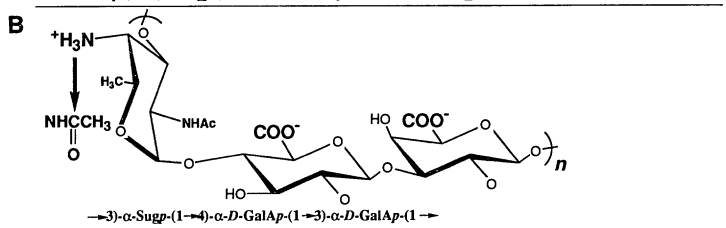
The chemically modified versions of polysaccharide A were tested in the rat model, and the AD_{50} values calculated (Table 1). Each of these polysaccharides induced fewer abscesses than the unmodified polysaccharide A. The AD_{50} for native polysaccharide A was less than 2 μg, whereas the AD_{50} values for polysaccharide A lacking a carboxyl group (negative charge) or an amino group (positive charge) were all greater than 200 μg. Modification of the charged groups reduced the biologic potency by at least two orders of magnitude, strongly suggesting that polysaccharide A requires both amino (positive) and carboxyl (negative) groups to promote abscess induction in this animal model. Each of the modifications yielded a significant reduction in abscess-inducing ability as compared with that of unmodified polysaccharide A ($P < 0.0005$). Similar chemical modifications made to the positive and negative charges on polysaccharide B (Fig. 1B) also resulted in polysaccharides with reduced abscess-inducing ability (*14*).

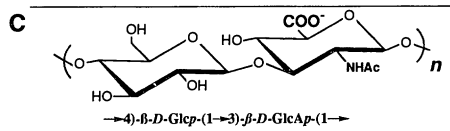
We next tested other bacterial polysaccharides with both positively and negatively











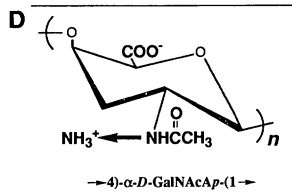


Fig. 1 (top). The fine structures of the *B. fragilis* polysaccharide repeating units, which were chemically modified (boxed roman numerals) to eliminate charged groups. (**A**) Polysaccharide A has one positive and one negative charge per repeating unit and was modified as follows: I, carbodiimide reduction of the negatively charged carboxyl group to a hydroxymethyl group; II, *N*-acetylation of the free amino group conferred by the trideoxyfucosamine; and III, deamination of the free amino group. (**B**) Polysaccharide B has one positively and two negatively charged groups and was modified as follows: I, carbodiimide reduction of the negatively charged carboxyl group to a hydroxymethyl group; II, *N*-acetylation of the free amino group on the 2-aminoethylphosphonate group; and III, de-*N*-acetylation of the three amino sugars, converting *N*-acetyl groups [designated (NHAc)] to free amino groups. **Fig. 2 (bottom).** Repeating unit structures of bacterial polysaccharides tested in the rat model. (**A**) C substance has three positively charged groups and two negatively charged groups per repeating unit. (**B**) The type 1 capsule from *S. pneumoniae* has one positive and two negative charges per repeating unit. For some experiments, the free amino group was neutralized by *N*-acetylation of the amino function (large arrow). (**C**) The type 3 capsule from *S. pneumoniae* has one negatively charged group per repeating unit. (**D**) The Vi polysaccharide from *S. typhi* is a homopolymer of aminuronic acid. This polysaccharide was selected for use because removal of the acetyl group after alkali treatment leaves a free amino group and gives this repeating unit one positively charged group and one negatively charged group (large arrow).

charged groups for activity in the rat model. Although the structures of hundreds of bacterial polysaccharides have been elucidated, most do not contain positively charged groups but rather have constituent monosaccharides that are neutral or negatively charged. Among the few natural polysaccharides that have oppositely charged groups, C substance, the group polysaccharide from *Streptococcus pneumoniae*, and the capsular polysaccharide of *S. pneumoniae* type 1 strains (*15, 16*) were selected for testing (Fig. 2).

C substance has a tetrasaccharide repeating unit with a total of three positive charges (conferred by a phosphatidylcholine substituent and two free amino groups) and two negative charges (conferred by phosphate groups) (Fig. 2A). The capsule of *S. pneumoniae* type 1 has a trisaccharide repeating unit with one positive charge (free amino group) and two negative charges (carboxyl groups) (Fig. 2B). As our hypothesis predicted, each was a potent inducer of intra-abdominal abscesses. The AD_{50} values of C substance and *S. pneumoniae* type 1 capsule were 5 and 31 μg, respectively (Table 2). Chemically modified *S. pneumoniae* type 1 polysaccharide, in which the free amino group was neutralized by *N*-acetylation (Fig. 2B), showed a marked reduction in activity (AD_{50} > 200 μg, P = 0.018 as compared with the *S. pneumoniae* type 1 capsule) (Table 2).

Bacterial polysaccharides that have repeating unit structures devoid of charged groups or that have one negatively charged group (carboxyl group) per repeating unit were also tested in the rat model. The capsular polysaccharide of *S. pneumoniae* type 3 (Fig. 2C) (*17*), a disaccharide repeating unit with one negative charge, was a poor inducer of abscesses in these animals (AD_{50} > 200 μg, $P < 0.005$ as compared with the *S. pneumoniae* type 1 CP) (Table 2). The capsular polysaccharide from *S. pneumoniae* type 14 (*18*), which has no charged groups, did not induce abscesses, even at high concentrations (200 μg). Capsular polysaccharides of group B *Neisseria meningitidis* (*19*) or of types Ia and III of group B streptococci (*20, 21*)—all with one negative charge per repeating unit—were poor inducers of abscesses (Table 2).

Finally, we tested whether we could "activate" an inactive polysaccharide that naturally has only one negative charge per repeating unit by converting it to a structure with both positive and negative charges. For this experiment, we selected the capsular polysaccharide from *Salmonella typhi*, a homopolymer of galactaminuronic acid, termed Vi antigen (Fig. 2D) (*22*). Vi antigen has an *N*-acetyl group at the C-2 position of the pyranose ring and a negatively charged carboxyl group at the C-6 position. The *N*-acetyl group was chemically cleaved by treatment with alkali (*23*), and the de-*N*-acetylation was confirmed by NMR spectroscopy. This treatment created a repeating unit with a positively charged free amino group and a negatively charged carboxyl group. Assessment of the unmodified and chemically modified Vi polysaccharides in the rodent model demonstrated that this chemical modification transformed the antigen into an abscess-inducing polysaccharide. The AD_{50} for the modified Vi polysaccharide was 16 μg, whereas the unmodified Vi had no abscess-inducing activity ($P < 0.005$) (Table 2).

Few structure-function relations have been defined for pathogenic microbes. This is especially true of polysaccharide antigens (*24*). Our finding that the *B. fragilis* CPC comprises two polysaccharides, each with unusual charged groups as part of their repeating unit structure, enabled us to investigate structural features of these carbohydrates that modulate a specific biologic function. The data presented here show that abscess induction in the peritoneal cavity of rodents is mediated by oppositely charged groups on bacterial polysaccharides and delineate one mechanism by which this host response occurs.

Table 2. Abscess induction by charged and uncharged bacterial polysaccharides. Rats were administered 10-fold dilutions of each of the polysaccharides mixed 1:1 with adjuvant. Data were accumulated from two separate experiments. Experiment 1: Pneumococcal polysaccharides were tested in the rodent model (structures shown in Fig. 2, A to C). Modification of the *S. pneumoniae* type 1 polysaccharide is shown in Fig. 2B. The AD_{50} and P values were calculated with the use of a mathematical model based on logistic regression analysis (*26*). Experiment 2: Control polysaccharides that lack charge or have negative charges were tested at highest dose only (200 μg). ND, not done. Experiment 3: Unmodified and modified versions of Vi antigen were tested (structure shown in Fig. 2D). The AD_{50} for de-*N*-acetylated Vi antigen was calculated by the method of Reed and Muench (*25*). ND, not done.

Polysaccharide	Fraction of rats with abscesses at a dose of			AD_{50} (μg)	P
	200 μg	20 μg	2 μg		
	Experiment 1				
C substance	17/18	12/18	6/19	5	—
S. pneumoniae type 1	17/20	7/18	2/18	31	—
S. pneumoniae type 1 (*N*-acetylated)	6/19	6/20	3/20	>200	0.018*
S. pneumoniae type 3	0/14	2/10	1/9	>200	<0.005*
	Experiment 2				
Group B meningococcal	1/10	ND	ND	—	—
Group B streptococcal type Ia	0/10	ND	ND	—	—
Group B streptococcal type III	1/10	ND	ND	—	—
S. pneumoniae type 14	2/10	ND	ND	—	—
	Experiment 3				
Vi antigen	3/20	ND	ND	—	—
Vi antigen (de-*N*-acetylated)	15/20	9/18	7/20	16	<0.005†

*As compared with type 1. †As compared with unmodified Vi antigen at a dose of 200 μg by Fisher's exact test.

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12. We used the rat model of intra-abdominal sepsis developed by Onderdonk (*4*). Male Wistar rats (180 to 200 g; Charles River Laboratories, Wilmington, MA) were used for all experiments. Animals were housed separately and received Chow (Ralston Purina, St. Louis, MO) and water ad libitum. Animals were anesthetized with a single intraperitoneal injection of 0.15 ml of Nembutal (50 mg/ml; Abbott Laboratories, North Chicago, IL), and their abdomens were shaved and swabbed with a tincture of iodine. An anterior midline incision (1 cm) was made through the abdominal wall and peritoneum, and a gelatin capsule containing 0.5 ml of inoculum was inserted into the pelvis. The incisions were closed with interrupted 3.0 silk sutures, and the animals were returned to the cages. The inoculum contained a 1:1 mixture of the test polysaccharide and an adjuvant solution containing sterile rat cecal contents and 10% barium sulfate (w/v). Six days later, animals were necropsied in a blinded fashion and examined for the formation of one or more intra-abdominal abscesses. Animal care was in accordance with the institutional guidelines set forth by Brigham and Women's Hospital and Harvard Medical School.
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26. A mathematical model was devised (i) to compare the biologic activities of modified and unmodified polysaccharides over a range of three doses (200, 20, and 2 μg) and (ii) to calculate AD_{50} values for each polysaccharide type. The general mathematical model, which is based on logistic regression analysis, was $\log[p/(1-p)] = \alpha + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4$ where p is the probability of abscess formation, α is the intercept, and $x_1 = \log(\text{dose}/20)$; $x_2 = 1$ if in the first modified polysaccharide group tested, otherwise $x_2 = 0$; $x_3 = 1$ if in the second modified polysaccharide group tested, otherwise $x_3 = 0$; $x_4 = 1$ if in the third modified polysaccharide group tested, otherwise $x_4 = 0$; and β_1, β_2, β_3, and β_4 are the regression coefficients corresponding to x_1, x_2, x_3, and x_4. The AD_{50} estimates were obtained from $-\alpha/\beta_1$ if in the unmodified group, $-(\alpha + \beta_2)/\beta_1$ if in the first modified polysaccharide group tested, $-(\alpha + \beta_3)/\beta_1$ if in the second modified polysaccharide group tested, and $-(\alpha + \beta_4)/\beta_1$ if in the third modified polysaccharide group tested.
27. We thank H. Jennings for C substance and NMR analyses; J. Robbins for C substance and Vi polysaccharides; J. Pier, M. Wessels, and L. Paoletti for critically reviewing the manuscript; R. Smith for technical assistance; and M. Goetsch for statistical analyses. Supported by grant 2T32AI07061-11AI and National Research Service Award grant 1F32 AI 084901 AI (to A.O.T.) from the National Institutes of Allergy and Infectious Diseases, National Institutes of Health.

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Converting Tissue Plasminogen Activator to a Zymogen: A Regulatory Triad of Asp-His-Ser

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Unlike most serine proteases of the chymotrypsin family, tissue-type plasminogen activator (tPA) is secreted from cells as an active, single-chain enzyme with a catalytic efficiency only slightly lower than that of the proteolytically cleaved form. A zymogenic mutant of tPA has been engineered that displays a reduction in catalytic efficiency by a factor of 141 in the single-chain form while retaining full activity in the cleaved form. The residues introduced in the mutant, serine 292 and histidine 305, are proposed to form a hydrogen-bonded network with aspartate 477, similar to the aspartate 194–histidine 40–serine 32 network found to stabilize the zymogen chymotrypsinogen.

Proteases are normally synthesized and secreted as zymogens that must be proteolytically cleaved to display their full enzymatic activity. The increase in catalytic efficiency (measured by k_{cat}/K_m, where k_{cat} is the rate of catalysis and K_m is the Michaelis constant) after cleavage can be dramatic. For example, the value of k_{cat}/K_m for the prototypical serine proteases chymotrypsinogen and trypsinogen increases by a factor of 10^4 to 10^6 immediately after cleavage of a single peptide bond (*1*).

A few serine proteases are unusual in that they are secreted from cells not as inactive precursors but as single-chain polypeptides that display high levels of enzymatic activity. For example, tPA is secreted as a 527–amino acid polypeptide that efficiently converts the plasma-borne zymogen plasminogen into the active protease plasmin, which then degrades the fibrin meshwork of thrombi (*2*). Plasmin also cleaves single-chain tPA into a two-chain form whose catalytic efficiency is certainly increased relative to the single-chain form, but only by a very modest amount.

Tissue-type plasminogen activator has been widely used as a therapeutic agent for the treatment of acute myocardial infarction. However, the systemic activity of clinical doses of tPA causes a significant depletion of circulating fibrinogen in patients who receive the drug (*3*), and a small minority of patients suffer severe hemorrhagic complications. These problems might be reduced if zymogen-like variants of tPA (zymogenic tPA) were available. In such cases, the catalytic activity of the circulating single-chain form of zymogenic tPA would be greatly diminished; however, once attached to the fibrin meshwork of a thrombus, zymogenic tPA could be cleaved by plasmin generated locally at the clot by the patient's own tPA and would then display full catalytic activity. We therefore investigated whether tPA could be converted into a zymogen by replacing key amino acids with residues that stabilize the zymogen forms of other serine proteases.

The serine protease domains of tPA and chymotrypsin have a 40% sequence identity; thus, comparing the sequence of tPA with the structure of chymotrypsinogen, we identified residues of tPA that are likely to contribute to the constitutive activity of single-chain tPA. The active conformation of members of the chymotrypsin family is secured by an ion pair between the ammonium ion of the mature NH_2-terminus and the carboxylate of Asp^{194} (*4*), an invariant residue that is flanked by amino acids that form the oxyanion hole (*5*) (Fig. 1). Although the zymogens chymotrypsinogen (*4*, *6*) and trypsinogen (*4*, *7*) differ in structure, both exhibit an altered conformation in which the side-chain of Asp^{194} is stabilized by an ion pair with a buried histidine, His^{40}. His^{40} also forms a hydrogen bond with Ser^{32} (*4*), which creates a triad (Asp^{194}-His^{40}-Ser^{32}) whose members are linked by ionic bonds (Fig. 1). The geometry of this "zymogen triad" found in chymotrypsinogen is very similar to that of the familiar catalytic triad, which consists of the same three residues.

In contrast to chymotrypsinogen and trypsinogen, tPA lacks the zymogen triad. Phe^{305} and Ala^{292} in tPA occupy positions that are homologous to His^{40} and Ser^{32} of chymotrypsin. To assess whether zymogen status and activation could be conferred on tPA, we used oligonucleotide-directed, site-specific mutagenesis to construct complementary DNAs (cDNAs) encoding the enzymes tPA(F305H) and tPA(A292S,F305H) (*8*). The mutated cDNAs were ligated into the SV40-based, transient expression vector pSVT7(RI−), and the resulting constructs were used to transfect COS-1 cells by the DEAE-dextran method (*9*). Enzymes were harvested from serum-free media, and their concentration was measured by solid-phase radioimmunoassay.

The activity of both the single- and two-chain forms of tPA, tPA(F305H), and tPA(A292S,F305H) toward the synthetic substrate methylsulfonyl-D-cyclohexyltyrosylglycyl-arginine-*p*-nitroaniline acetate was measured as previously described (*10*). Data from these assays allow (Table 1) several conclusions. (i) The catalytic efficiency of the two-chain form of both tPA(F305H) and

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