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# Selective Chemical Catalysis by an Antibody

SCOTT J. POLLACK, JEFFREY W. JACOBS, PETER G. SCHULTZ

**The immunoglobulin MOPC167, which binds the transition state analog *p*-nitrophenylphosphorylcholine with high affinity, catalyzed the hydrolysis of the corresponding carbonate 1. MOPC167 catalysis displayed saturation kinetics with catalytic constant ($k_{cat}$) = 0.4 $min^{-1}$ and Michaelis constant ($K_m$) = 208 μ*M*, showed substrate specificity, and was inhibited by *p*-nitrophenylphosphorylcholine. The rate of the reaction was first order in hydroxide ion concentration between *p*H 6.0 and 8.0. The lower limit for the rate of acceleration of hydrolysis by the antibody above the uncatalyzed reaction was 770. This study begins to define the rules for the generation of catalytic antibodies.**

A KEY ELEMENT IN THE SYNTHESIS of enzymatic catalysts is the rational design of highly selective substrate binding sites. Current efforts in this regard focus primarily on two strategies: the synthesis of cavity-containing organic compounds and the modification of enzyme specificities by oligonucleotide-directed mutagenesis. With the advent of monoclonal antibodies, homogeneous ligand-binding sites with enzyme-like binding affinities and specificities can be generated for most biologically active macromolecules as well as smaller synthetic molecules. This property has made antibodies one of the most important classes of receptors in biology and medicine today, finding applications in diagnostics, drug delivery, and protein and nucleic acid purification and characterization. The development of strategies for introducing catalytic activity into the combining sites of immunoglobulins could potentially provide general routes for the construction of enzymatic catalysts with tailored specificities and catalytic properties.

One mechanism whereby enzymes are believed to act as highly specific catalysts is by providing an environment complementary in structure and electronic distribution to a rate-limiting transition state of a given reaction. This hypothesis is supported by crystal structures of a number of hydrolytic enzymes as well as by studies of enzyme inhibition by transition state analogs (*1, 2*). One might then expect that the highly specific binding interactions of antibodies could be exploited to catalyze chemical reactions by a similar mechanism. An antibody elicited to a haptenic group resembling the presumed transition state of a given reaction should lower the free energy of activation by stabilizing the transition state relative to reactants or products (*3, 4*). Such an approach, in addition to affording selective catalytic antibodies, may provide additional insight into mechanisms of enzyme catalyzed reactions.

In order to determine the viability of this strategy, we and others (*5, 6*) have begun to investigate the catalytic properties of antibodies specific to transition state analogs for the hydrolysis of esters and carbonates. We chose these well-characterized reactions in order to simplify mechanistic studies of ligand binding and catalysis and because tetrahedral phosphonates and phosphonamidates have been reported to act as transition state analog inhibitors for enzymatic peptide hydrolysis (*7–10*). Our initial studies have therefore focused on immunoglobulins specific for phosphonate and phosphate tetrahedral transition state analogs. We have succeeded in characterizing two catalytic antibodies, one that hydrolyzes *p*-nitrophenyl *N*-trimethylammonioethyl carbonate and a second that hydrolyzes methyl-*p*-nitrophenyl carbonate. The latter catalytic antibody was generated by specifically eliciting monoclonal antibodies to the corresponding *p*-nitrophenyl phosphonate transition state analog (*6*). However, to begin to understand in detail the mechanism of antibody catalysis so that we can develop rules and guidelines for generating more sophisticated catalysts, we have focused our attention on the immunoglobulin A (IgA) MOPC167 (*11, 12*).

MOPC167 binds nitrophenylphosphorylcholine, a transition state analog for the hydrolysis of *p*-nitrophenyl *N*-trimethylammonioethyl carbonate chloride **1** with association constant ($K_a$) = $1.4 \times 10^6$ $M^{-1}$ (*13*) (Fig. 1). MOPC167 is a member of a well-characterized class of antibodies specific for phosphorylcholine (PC) mono- and diesters (*14–16*). These antibodies share a high degree of sequence homology among amino acids in the heavy chain (*16–18*), show a high affinity for phosphorylcholine and related ligands (*19, 20*), and can be obtained easily in large quantities. More-

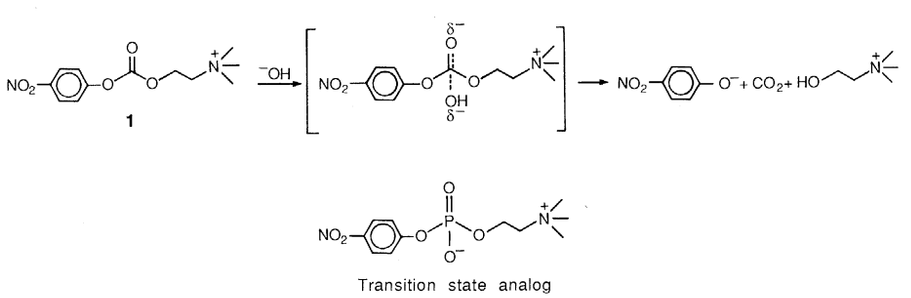


Fig. 1. Transition state analog for aqueous hydrolysis of carbonate **1**.

Department of Chemistry, University of California, Berkeley, CA 94720.

over, the three-dimensional structure of a Fab'-PC complex of a typical member of this group, McPC603, has been reported (*21–23*). The tetrahedral phosphate in the McPC603-PC complex is stabilized by hydrogen bonding and electrostatic interactions with $Tyr^{33H}$, $Arg^{52H}$, and (to some extent) with $Lys^{54H}$ (*23*). These residues are conserved in the PC-binding antibodies (*17, 23*), suggesting that the combining site of MOPC167 should be complementary to the transition state for hydrolysis of carbonate **1**. The trimethylammonium ion in the McPC603-PC complex is stabilized by interactions with conserved heavy-chain (H) residues $Glu^{35H}$ and $Glu^{59H}$, and light-chain (L) residue $Asp^{91L}$ (*23*). Moreover, the x-ray structure suggests (*23*) that the carbonyl group in a MOPC167-substrate complex should be accessible to attack by an external nucleophile such as water or hydroxide ion.

We report here the selective complex formation and catalytic turnover of substrate by immunoglobulin MOPC167 under physiological conditions. MOPC167 (1.6 g) (*24*) was purified from 50 ml of ascites fluid by mild reduction, followed by alkylation and affinity chromatography on glycyltyrosine-(*p*-azophenylphosphorylcholine)–derivatized CL Sepharose 4B (*25*). Affinity-purified antibody was then dialyzed exhaustively against assay buffer and judged to be homogeneous by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (*26*) (Fig. 2).

The rates of hydrolysis of carbonate **1** in the presence ($k_{obs}$) and absence ($k_{un}$) of 11.6 μ*M* MOPC167 were determined as a function of substrate concentration. Carbonate hydrolysis was monitored spectrophotometrically by the increase in absorbance at 400 nm due to nitrophenolate ion release. Immunoglobulin MOPC167 was found to catalyze the hydrolysis of carbonate **1** with kinetics consistent with the Michaelis-Menten rate expression:

$$V = \frac{k_{cat}[Ig][\mathbf{1}]}{K_m + [\mathbf{1}]}$$

$$[Ig] + [\mathbf{1}] \rightleftarrows [Ig \cdot \mathbf{1}] \xrightarrow{k_{cat}} [Ig] + \text{products}$$

The value of the catalytic constant ($k_{cat}$) and the Michaelis constant ($K_m$) were found to be $0.4 \pm 0.04$ $min^{-1}$ ($\bar{x} \pm SD$) and $208 \pm 43$ μ*M* (*27*), respectively (Fig. 3).

The following controls were carried out. The catalytic activity associated with MOPC167 was destroyed by thermal denaturation of the antibody at 95°C for 10 minutes followed by rapid cooling to 0°C. MOPC167 was also cleaved with pepsin (*28*) and the resulting Fab fragments were isolated by affinity chromatography as described above. The Fab fragment, which

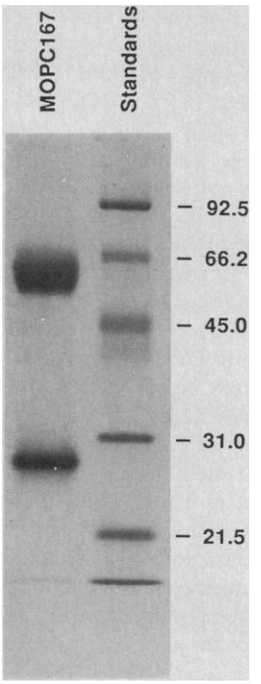


Fig. 2. Coomassie blue–stained 10% SDS-polyacrylamide gel of immunoglobulin MOPC167 purified by affinity chromatography. MOPC167 was overloaded (20 μg) to reveal minor impurities (*26*).

contains the antibody combining site, accelerated the rate of hydrolysis of carbonate **1** to the same degree as native Ig. In addition, we examined the rate of hydrolysis of carbonate **1** in the presence of 12 μ*M* of a purified IgG1 antibody to staphylococcal enterotoxin B (*29*). As expected, this antibody, which has no appreciable binding affinity for nitrophenylphosphorylcholine, did not accelerate the rate of hydrolysis of carbonate **1** above the background rate. Moreover, MOPC167 did not accelerate the rate of hydrolysis of the neutral substrate, dinitrophenyl carbonate, above the background rate. Finally, modification of MOPC167 with the Tyr-specific nitrating reagent, tetranitromethane, abolished all catalytic activity (*30, 31*), suggesting the presence of a Tyr at the catalytic site. These results are consistent with the selective catalytic hydrolysis of carbonate **1** by the immunoglobulin MOPC167. Moreover, MOPC167 displays saturation kinetics, demonstrating the reversible formation of a substrate-antibody complex followed by intracomplex catalysis (with rate constant $k_{cat}$) and release of product. In summary, the conclusion that catalysis is associated with the antibody combining site is based on the following: (i) catalysis appears to depend on the presence of the positively charged choline moiety, (ii) noncholine-specific Ig does not accelerate hydrolysis, and (iii) the catalytic site is contained within the Fab fragment and subject to inactivation with tetranitromethane.

The MOPC167-catalyzed hydrolysis of carbonate **1** was inhibited by the addition of the transition state analog, *p*-nitrophenylphosphorylcholine. The inhibition constant ($K_i$) for the formation of the MOPC167-nitrophenylphosphorylcholine complex was determined by measuring the rate of hydrolysis of 100 μ*M* carbonate **1** in the presence of 11.6 μ*M* MOPC167 at varying inhibitor concentrations. A Dixon plot (*32*) afforded a $K_i$ of $5 \pm 1.5 \times 10^{-6}M$ at 30°C in 5 m*M* tris HCl. This value is close to the $K_a$ of $1.4 \times 10^{6}M^{-1}$ for the association of nitrophenylphosphorylcholine with MOPC167 at 5°C in borate-cacodylate–buffered saline, *p*H 7.0 (*13*). MOPC167 was also treated with the irreversible affinity label, *p*-diazonium phenylphosphorylcholine, which has been used to affinity-label the combining site of the phosphorylcholine-specific Ig T15 (*25, 33*). Reaction of 58 μ*M* MOPC167 with three equivalents of *p*-diazonium phenylphosphorylcholine at 0°C in 0.16*M* NaCl, 0.2*M* borate buffer, *p*H 8.3, followed by extensive dialysis resulted (*33*) in complete loss of the catalytic activity of MOPC167. Again, these results are consistent with catalysis occurring in the combining site of MOPC167.

If the role of the antibody combining site is to stabilize the transition state formed by attack of an external nucleophile on Ig-complexed carbonate **1**, then it would be reasonable to expect a first-order dependence of the rate of hydrolysis of carbonate **1** on hydroxide ion concentration. The *p*H dependence of the hydrolysis of carbonate **1**

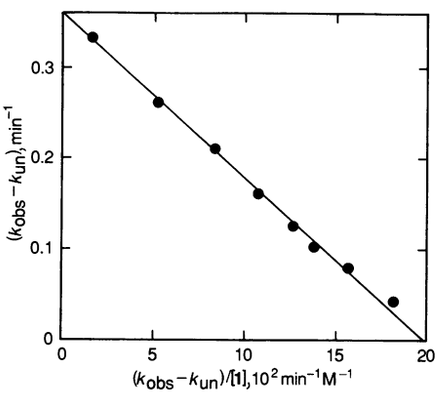


Fig. 3. Eadie (*36*) plot of $(k_{obs}-k_{un})$ for hydrolysis of carbonate **1** as a function of $(k_{obs}-k_{un})/[\mathbf{1}]$. Measurements in 5 m*M* tris HCl, *p*H 7.0, at 30°C in the presence of 11.6 μ*M* MOPC167. Protein molarity was determined by absorbance at 280 nm with the extinction coefficient ($E^{0.1\%}_{1cm}$) = 1.37 and a molecular weight of 150,000 for IgA. Reactions were initiated by addition of 10 μl of a stock substrate solution ($CH_3CN$) to 0.5 ml equilibrated reaction medium, containing antibody. Kinetic constants were determined by the method of initial rates.

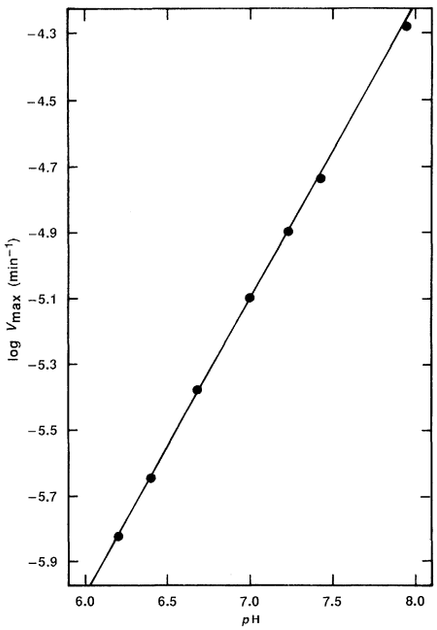


Fig. 4. Plot of log $V_{max}$ as a function of *p*H. Measurements in 25 m*M* tris HCl at 30°C in the presence of 11.6 μ*M* MOPC167, 2 m*M* carbonate **1**. The *p*H remained unchanged before and after rate measurements.

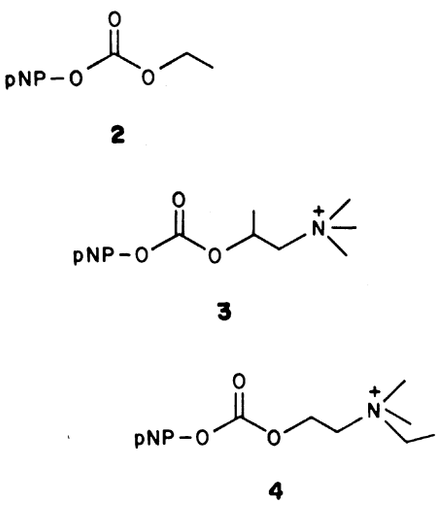


Fig. 5. Nitrophenyl carbonates used to determine the structural specificity of the antibody.

was examined in the presence of 11.6 μ*M* MOPC167 between *p*H 6.0 and 8.0 in 25 m*M* tris HCl at 30°C. The $V_{max}$ of the MOPC167-catalyzed reaction exhibited a first-order dependence on hydroxide ion concentration in this *p*H range (Fig. 4), whereas $K_m$ remained between 200 and 250 μ*M* (consistent with the *p*H dependence of $K_a$ for nitrophenylphosphorylcholine reported by Goetz and Richards (*13*)). The role of hydroxide ion may either be to directly hydrolyze the complexed carbonate or to hydrolyze an intermediate covalent choline acyl-antibody adduct.

Since the substrate can also be cleaved in the absence of Ig by direct nucleophilic attack of hydroxide ion, we can compare this rate, $v_{uncat} = k_{uncat}$ [**1**][OH$^-$], with the rate of hydrolysis in the MOPC167-substrate complex, $v_{complex} = k_{complex}$ [complex] [OH$^-$]. The value of $k_{uncat}$ [OH$^-$] was determined to be $5.2 \times 10^{-4}$ min$^{-1}$ at 30°C in 5 m*M* tris HCl, *p*H 7.0, by extrapolation of the rate of the uncatalyzed reaction to zero buffer concentration. Therefore, the ratio of $k_{cat}/k_{uncat}$, which reflects the acceleration of hydrolysis by the antibody combining site is 770 per antibody. Moreover, this ratio may be considered a lower limit, since the rate of hydrolysis of choline carbonate **1** is 15-fold higher than that of uncharged ethyl nitrophenyl carbonate in the absence of MOPC167. The mechanism for this acceleration, which has been attributed to intramolecular assistance of the charged trimethylammonium ion in the case of the corresponding ester, may not be operating in the antibody-substrate complex (*34*).

In light of the high degree of structural specificity displayed by antibodies, we have investigated the rates of hydrolysis of nitrophenyl carbonates **2**, **3**, and **4** by MOPC167 (Fig. 5). MOPC167 did not catalyze the hydrolysis of neutral carbonate **2** to any appreciable extent. The rate and Michaelis constants for substrates **3** and **4** at 30°C in 5 m*M* tris HCl, *p*H 7.0, were as follows: $k_{cat}$(**3**) = 0.2 ± 0.04 min$^{-1}$, $K_m$(**3**) = 2 ± 0.5 m*M* and $k_{cat}$(**4**) = 1.07 ± 0.15 min$^{-1}$, $K_m$(**4**) = 650 ± 120 μ*M*. As expected (*19*), the choline moiety is an essential substrate recognition element. Removal of the positively charged ammonium ion, modification of an *N*-methyl substituent to *N*-ethyl, or introduction of a methyl group into the methylene side chain led to increases in substrate $K_m$. These results are consistent with the high degree of binding specificity displayed by antibodies (*35*).

Although we have demonstrated that an antibody can function as a selective catalyst, the mechanism of catalysis remains unresolved. One possibility is that hydrolysis involves a single chemical step in which the antibody combining site acts to stabilize the transition state, relative to substrate or product, for attack of a binding-site accessible hydroxide ion on carbonate **1**. The conserved amino acids, Arg$^{52H}$, Tyr$^{33H}$, and Lys$^{54H}$, which are in close proximity to the bound phosphate in the McPC603-PC complex, may serve such a function. This mechanism is consistent with the observation that MOPC167 binds the charged tetrahedral transition state analog, *p*-nitrophenylphosphorylcholine, with higher affinity (*27*) than the planar carbonate **1**; the mechanism also is consistent with the first-order dependence of the reaction on hydroxide ion concentration.

Alternatively, an active-site amino acid side chain may initially attack the antibody-complexed carbonate, followed by cleavage of the covalent antibody adduct by a binding site–accessible hydroxide ion in the rate-determining second step. A candidate for the reactive side chain in MOPC167 is the conserved Tyr$^{33H}$, which is hydrogen-bonded to the phosphate monoester in McPC603; or Asp$^{100H}$, which has also been postulated to be in close proximity to the bound phosphate in MOPC167 (*23*). Catalysis in this case may be due to complexation of substrate in close proximity to the nucleophilic group. Sequence data are available for more than 25 phosphorylcholine-binding antibodies derived from the same variable region H-chain germline gene but differing in their binding affinities for phosphorylcholine and acetylcholine esters (*16–18*). A systematic investigation of the catalytic properties of these proteins should provide us with a unique opportunity to probe the structure-function relationships leading to antibody catalysis.

In conclusion, the work described here is a first step toward defining the rules and strategies whereby catalytic activity can be introduced into antibodies. It should be possible to extend these ideas to more complex systems (such as selective peptide hydrolysis) and to generate catalytic antibodies by chemical derivatization of antibodies with catalytic functionalities such as cofactors or metals. Together, these approaches may enable us to tailor-make catalysts for use as tools in biology, chemical synthesis, and medicine.

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# Lipoprotein Mutations in Pigs Are Associated with Elevated Plasma Cholesterol and Atherosclerosis

JAN RAPACZ, JUDITH HASLER-RAPACZ, KATHERINE M. TAYLOR, WILLIAM J. CHECOVICH, ALAN D. ATTIE

**A strain of pigs bearing three immunogenetically defined lipoprotein-associated markers (allotypes), designated Lpb5, Lpr1, and Lpu1, has marked hypercholesterolemia on a low fat, cholesterol-free diet. Unlike individuals with familial hypercholesterolemia or WHHL rabbits, the affected pigs have normal low density lipoprotein receptor activity. The animals, by 7 months of age, have extensive atherosclerotic lesions in all three coronary arteries. This strain of pig represents an animal model for atherosclerosis and hypercholesterolemia associated with mutations affecting the structures of plasma lipoproteins. One of the variant apolipoproteins, Lpb5, is apolipoprotein-B. A second variant apolipoprotein (Lpr1), termed apo-R, is a 23-kilodalton protein present in both the very low density ($d < 1.006$ g/ml) and the very high density ($d > 1.21$ g/ml) fractions of pig plasma. Isoforms of this protein correlate with two *Lpr* alleles, $Lpr^1$ and $Lpr^2$. The *Lpr* genes segregate independently of the $Lpb^5$ and $Lpu^1$ alleles. The Lpu1 allotype is a component of low density lipoprotein and is genetically linked to $Lpb^5$.**

CORONARY HEART DISEASE IS THE leading cause of death in the United States as well as in some other countries. Although epidemiological studies and studies in research animals have revealed as causes a number of environmental risk factors, they also disclose a strong genetic contribution to the risk of developing the disease (*1*). Several mutations predisposing individuals to atherosclerosis have been identified. For example, familial hypercholesterolemia (FH) is a disease caused by mutations affecting the receptor for low density lipoprotein (LDL) (*2*). The WHHL rabbit also has an inborn deficiency of LDL receptor activity and develops premature atherosclerosis (*3*). FH is the most common known inherited metabolic disease associated with premature atherosclerosis in humans, but FH and the other known mutations are rare relative to the high incidence of atherosclerosis in the human population. It is therefore likely that other mutations may exist which lead to premature atherosclerosis. We now report an association between mutations in plasma lipoproteins and atherosclerosis in a strain of pigs.

J. Rapacz and J. Hasler-Rapacz, Department of Genetics and Department of Meat and Animal Science, College of Agricultural and Life Sciences, University of Wisconsin–Madison, Madison, WI 53706.
K. M. Taylor, Department of Surgery, School of Medicine, University of Wisconsin–Madison, Madison, WI 53706.
W. J. Checovich, Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin–Madison, Madison, WI 53706.
A. D. Attie, Department of Biochemistry, College of Agricultural and Life Sciences, and Department of Comparative Biosciences, School of Veterinary Medicine, University of Wisconsin–Madison, Madison, WI 53706.

The principal bloodborne transporter of cholesterol in many animal species, including human and pig, is LDL. Progress in the biochemical identification of mutations in the protein moiety of LDL has been hindered by its unusual physical properties (*4*). Apo-B, which makes up more than 90 percent of LDL protein, is difficult to solubilize by conventional detergent solubilization techniques used to study membrane proteins. The protein has a molecular size of 514 kD (*4, 5*).

Although apo-B has been resistant to biochemical analysis, valuable information has been obtained through immunogenetic investigation. Polyclonal alloimmune antibodies have been used to identify polymorphism in cattle, carp, chicken, human, mink, pig, rabbit, rhesus monkey, and sheep (*6*). Monoclonal antibodies identified three human apo-B markers encoded by three alleles (*7*) but no discernible clinical phenotypic differences were found between individuals bearing these different alleles.

In our studies lipoproteins obtained from pigs of various breed origins were used as alloantigens to obtain immune sera exhibiting different patterns of precipitation reactions with randomly selected pig sera. Genetic studies demonstrated that most of the epitopes associated with the LDL particles are inherited in groups of eight (*6, 8*). This led to the conclusion that the set of eight epitopes is encoded by a single codominant allelic apo-B gene. A total of eight different sets has been found. Each set has seven epitopes in common and differ from the other sets by one distinctive characterizing epitope. Thus there are a total of 16 epitopes, each defined by an alloantiserum. For clarity we have omitted the names of the

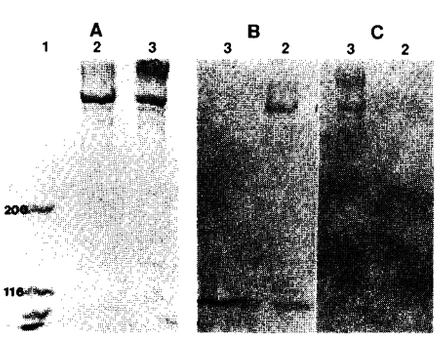


Fig. 1. Immunoblot of LDL's with antibodies to Lpb3 and antibodies to Lpb5. (Lane 1) Molecular size markers in kilodaltons; lane 2, LDL from an $Lpb^{1/3}$ pig; lane 3, LDL from an $Lpb^{5/5}$ pig. (A) Coomassie blue stain. (B) Immunoblot with antibodies to Lpb3. (C) Immunoblot with antibodies to Lpb5 (*20*).