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Conversion of the Noncooperative *Bacillus subtilis* Aspartate Transcarbamoylase into a Cooperative Enzyme by a Single Amino Acid Substitution†

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ABSTRACT: Allosteric enzymes are part of a unique class of enzymes which regulate metabolic pathways. On the molecular level, allosteric regulation is the result of interactions between discrete binding sites on the enzyme. In order to accommodate these multiple binding sites, allosteric enzymes have evolved with oligomeric quaternary structures. However, only a few oligomeric enzymes are known to have regulatory interactions between binding sites. Is regulatory activity an inherent property of oligomeric enzymes? The trimeric *Bacillus subtilis* aspartate transcarbamoylase catalyzes the first committed step of the pyrimidine biosynthetic pathway and is not known to be a regulatory enzyme. When an alanine residue is substituted for the active-site residue Arg-99 by site-specific mutagenesis, the regulatory activity of homotropic substrate cooperativity (Hill coefficient of 1.5) is observed in the resulting mutant enzyme. These results suggest that homotropic regulation may have evolved by a relatively small number of mutations to an oligomeric enzyme.

In the mechanism of allosteric regulation, the overall throughput or rate of a metabolic pathway is limited by the velocity of a key regulatory enzyme. The activity of this enzyme is in turn affected by two types of interactions between discrete binding sites: first, heterotropic interactions between active sites and allosteric binding sites, in which the binding of allosteric molecules can either enhance or diminish activity; second, homotropic interactions between active sites, in which cooperative substrate binding causes a sigmoidal dependence of activity upon substrate concentration. Two microscopic equilibrium models are commonly used to explain the physical basis of homotropic interactions in enzymes (Monod et al., 1965; Koshland et al., 1966). In these models, the enzyme exists in at least two conformations, which differ in catalytic

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activity and substrate affinity. Finally, regulatory enzymes also belong to a larger class of proteins, which have oligomeric or multidomain quaternary structures.

The oligomeric aspartate transcarbamoylase (EC 2.1.3.2) from *Escherichia coli* (*E. coli*) is used as a model system for the molecular mechanism of allosteric regulation (Kantrowitz & Lipscomb, 1988). This enzyme catalyzes the committed step of the pyrimidine biosynthetic pathway, the formation of *N*-carbamoyl-L-aspartate from carbamoyl phosphate and L-aspartate. The activity of the enzyme is feedback-inhibited by CTP and UTP, the end products of the pyrimidine biosynthetic pathway, and is activated by ATP, an end product of the purine biosynthetic pathway (Gerhart & Pardee, 1962; Wild et al., 1989). The enzyme also shows positive cooperativity for both substrates (Bethell et al., 1968). The native enzyme is composed of two catalytic trimers, each consisting of three identical 33 000-dalton polypeptide chains (catalytic chains), and three regulatory dimers, each consisting two identical 17 000-dalton polypeptide chains (regulatory chains). The amino acid sequence of both the catalytic and regulatory polypeptide chains has been determined (Konigsberg & Henderson, 1983; Weber, 1968). The isolated catalytic trimer does not show cooperativity for substrates and is insensitive to the allosteric molecules (Porter et al., 1969), while the isolated regulatory dimer binds ATP, CTP, and UTP but is devoid of catalytic activity (Gerhart & Schachman, 1965). The three-dimensional structure of the enzyme has been determined to atomic resolution in its unliganded form and in the presence of the following ligands: carbamoyl phosphate, ATP, CTP, the bisubstrate analogue *N*-phosphonacetyl-L-aspartate, the carbamoyl phosphate analogue phosphonoacetamide, the aspartate analogues succinate and malonate, and various combinations of the above (Ke et al., 1984, 1988; Krause et al., 1987; Kim et al., 1987; Gouaux & Lipscomb, 1988, 1990; Stevens et al., 1990).

The trimeric aspartate transcarbamoylase from *Bacillus subtilis*, unlike the *E. coli* enzyme, does not show cooperativity for either substrate, and the activity of the enzyme is insensitive to both pyrimidine and purine nucleotides (Brabson & Switzer, 1975). Although the *B. subtilis* aspartate transcarbamoylase is not an allosteric enzyme, it is developmentally regulated. Regulation occurs in the stationary bacterial growth phase prior to sporylation, where the enzyme is degraded by an ATP-dependent protease (Maurizi & Switzer, 1978). The amino acid sequence of the 33 500-dalton polypeptide chain has been determined and shows 35% sequence homology with the catalytic chain of the *E. coli* enzyme (Lerner & Switzer, 1986). Furthermore, the native *B. subtilis* enzyme, like the *E. coli* catalytic subunit, is a trimer consisting of three identical polypeptide chains (Brabson & Switzer, 1975). Recently, the X-ray structure of the *B. subtilis* enzyme was determined at 3-Å resolution (Stevens et al., 1991). The overall structure is more similar to that observed for the *E. coli* catalytic subunit in the T rather than the R allosteric state.

In a previous study of the *E. coli* enzyme, a mutant version of the enzyme with an alanine substituted for an arginine at position 105 in the catalytic chain was generated by site-specific mutagenesis (Stebbins et al., 1989). From the three-dimensional structure of the *E. coli* enzyme in the presence of carbamoyl phosphate and succinate (Figure 1), the side chain of Arg-105_{EC}[1] interacts with the carbonyl oxygen and a terminal phosphate oxygen of carbamoyl phosphate as well as a carboxylate oxygen of succinate (Gouaux & Lipscomb, 1988). Analysis of the Arg-105→Ala_{EC}[2] enzyme showed that the interactions involving the side chain of Arg-105_{EC}, as seen in the three-dimensional structure, are important for the binding of both substrates and for catalysis (Table I). The side chain of Arg-105_{EC} most likely facilitates catalysis by bringing the two substrates in close proximity in the transition state and by polarizing the carbonyl group of carbamoyl phosphate to make it more susceptible to nucleophilic attack. One of the most striking results from this earlier study was the cooperativity observed in the aspartate saturation curve of the isolated Arg-105→Ala_{EC} catalytic trimer (Table I) (Stebbins et al., 1989). The aspartate cooperativity, which is normally seen in the native *E. coli* enzyme, is an inherent property of the catalytic trimer and not a separate phenomenon. Thus, the catalytic trimer can be converted into its allosteric form either by the association of the regulatory

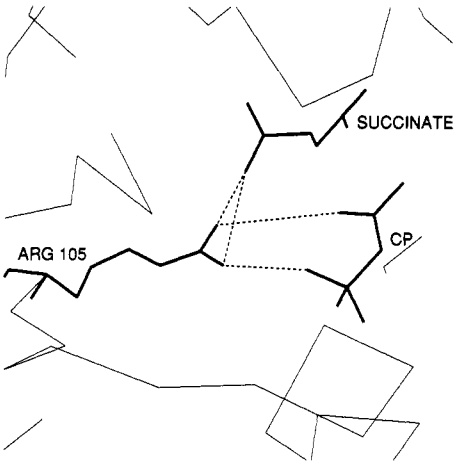

FIGURE 1: Active-site region of *E. coli* aspartate transcarbamoylase near the residue Arg-105 (Gouaux & Lipscomb, 1988). The amino acid side chain of Arg-105, the substrate carbamoyl phosphate, and the inhibitor succinate are shown in boldface. As indicated by the dashed lines, the guandinium group of Arg-105 interacts with a terminal phosphate oxygen and the carbonyl oxygen of carbamoyl phosphate as well as a carboxylate oxygen of succinate.

Table I: Kinetic Parameters for Wild-Type and Mutant Aspartate Transcarbamoylases[a]

enzyme	max velocity[b]	$[S]_{0.5}^{Asp}$ (mM)	n_{Hill}^{Asp}	$[S]_{0.5}^{CP}$ (mM)
wild-$type_{BS}$	13.0	5.3	1.0	0.04
Arg-99→Ala_{BS}	1.2	750	1.5	2.9
wild-type catalytic $trimer_{EC}$[c]	25.9	6.7	1.0	0.02
Arg-105→Ala catalytic $trimer_{EC}$[c]	0.15	450	2.0	23.0

[a] These data were determined from either aspartate or carbamoyl phosphate saturation curves, that were fit by nonlinear least squares to either the Michaelis–Menten equation or the Hill equation, that incorporated a term for substrate inhibition when necessary (Pastra-Landis et al., 1978). Colorimetric assays were performed in 0.05 M Tris–acetate buffer (pH 8.3) at 25 °C, holding the concentration of the nonvaried substrate constant and saturating (Pastra-Landis et al., 1981). [b] The maximal velocity represents the maximal observed specific activity, millimoles of *N*-carbamoyl-L-aspartate formed per hour per milligram of protein, of the aspartate saturation curves. [c] The data for the *E. coli* catalytic trimer were previously reported (Stebbins et al., 1989).

[1] Abbreviations: subscript EC, *Escherichia coli*; subscript BS, *Bacillus subtilis*; $[S]_{0.5}$, substrate concentration required for half the maximal observed velocity.

[2] The notation used to name mutant enzymes is, for example, the Arg-105→Ala_{EC} enzyme. The wild-type amino acid and location within the polypeptide chain are indicated to the left of the arrow while the new amino acid is indicated right of the arrow.

dimers and a second catalytic trimer, as in the native *E. coli* enzyme, or by mutagenesis, as in the Arg-105→Ala_{EC} enzyme.

In order to determine whether this is a more general phenomenon, the noncooperative trimeric *B. subtilis* aspartate transcarbamoylase was studied. Comparison of the amino acid sequence, between the catalytic chain of the *E. coli* enzyme and the polypeptide chain of the *B. subtilis* enzyme, revealed that Arg-99_{BS} is homologous to Arg-105_{EC} (Lerner & Switzer, 1986). Thus, a mutant version of the *B. subtilis* enzyme was constructed with an alanine residue at position 99 by site-specific mutagenesis.

Materials and Methods

Materials. The bacterial strains MV1190 [Δ(*lac-proAB*), *supE*, Δ(*sri-recA*)306::Tn10(tet^r)/F′*traD36*, *proAB*, *lacI*^{q}ΔM15], CJ236 [*dut1*, *ung1*, *thi1*, *relA1*/pCJ105 (Cm^r)], and TB2 [Δ*argI-pyBI*] were generously provided by J. Messing, T. Kunkel, and J. Wild, respectively. The plasmids pUC119 and pLS2000 were generously provided by J. Messing and R. Switzer, respectively. All other materials are as previously described (Stebbins et al., 1989).

Construction of the Plasmid pEK151. In order to create mutant versions of the *B. subtilis* aspartate transcarbamoylase enzyme, the *B. subtilis* aspartate transcarbamoylase gene, *pyrB*, was introduced into the phagemid vector pUC119. This vector contains both a plasmid and an M13 origin of replication, which allows for the isolation of the single-stranded DNA used in site-specific mutagenesis (Vieira & Messing, 1987). Following restriction enzyme digestion and agarose gel electrophoresis, the *Xba*I to *Hin*dIII fragment of 1071 base pairs, which contained the *pyrB* structural gene, was isolated from the plasmid pLS2000 with glass beads. In addition, the *Xba*I to *Hin*dIII backbone fragment from the phagemid vector pUC119 was isolated in a similar fashion, following digestion with the same two restriction enzymes and treatment with alkaline phosphatase. The fragment containing the *pyrB* gene was combined with the fragment from pUC119 and treated with T4 DNA ligase. Selection for ampicillin resistance was accomplished by tranforming the mixture into competent MV1190 cells, which are incapable of recombination. Plasmid DNA was isolated by alkaline lysis (Maniatis et al., 1982) from 15 candidates, and 1 of these plasmids (pEK151) was determined to have the correct construction by restriction enzyme analysis.

Site-Specific Mutagenesis and Construction of pEK165. Specific base changes were introduced into the *pyrB* gene by the method of Kunkel (1985) with the following modification. The uridine-containing template was generated by a helper phage (M13KO7) coinfection of the bacterial strain CJ236, which was transformed with the phagemid vector pEK151. Following mutagenesis, single-stranded DNA from 10 candidates was isolated by M13K07 co-infection and sequenced by the dideoxy method. One of these candidates had the DNA sequence corresponding to the mutation, and plasmid DNA was isolated by alkaline lysis. Following restriction enzyme digestion and agarose gel electrophoresis, the *Sph*I to *Sac*I fragment of 657 base pairs, which contained the desired mutation, was isolated from this candidate by glass beads. In addition, the *Sph*I to *Sac*I fragment from the vector pLS2000, which contained the remainder of the *pyrB* gene, was isolated in a similar fashion, following digestion with the same two restriction enzymes. The fragment containing the desired mutation was combined with the fragment from pLS2000 and treated with T4 DNA ligase. Selection for ampicillin resistance was accomplished by transforming the ligation mixture into competent MV1190 cells. Following plasmid isolation by alkaline lysis, restriction enzyme analysis as well as plasmid sequencing by the dideoxy method was used to confirm both the mutation and the plasmid construction.

Overexpression of the Wild-Type and Arg-99→Ala B. subtilis Aspartate Transcarbamoylases. The wild-type and Arg-99→Ala *B. subtilis* aspartate transcarbamoylases were overexpressed by the method of C. Lerner, E. Ferguson, and R. Switzer (unpublished results) in the *E. coli* strain TB2 containing the plasmids pLS2000 and pEK165, respectively. The bacteria were grown with vigorous aeration in a medium consisting of Na_2HPO_4 (6 g/L), KH_2PO_4 (3 g/L), NaCl (0.5 g/L), NH_4Cl (1 g/L), casamino acids (2 g/L), L-arginine (0.05 g/L), $MgCl_2$ (0.2 g/L), $CaCl_2$ (0.015 g/L), ampicillin (50 μg/mL), and 0.5% glycerol. When the culture reached an absorbance of approximately 0.1 at 566 nm (approximately 6 h), isopropyl β-D-thiogalactopyranoside was added to a final concentration of 1 mM. The culture was harvested after approximately 20 h of total growth time.

Other Methods. Oligonucleotide synthesis, enzyme purification, determination of protein concentration, and the assays for aspartate transcarbamoylase were as previously described (Stebbins et al., 1989).

Results and Discussion

Kinetic Properties of the Wild-Type and Arg-99→Ala_{BS} Enzymes. As seen in Table I, the Arg-99→Ala_{BS} enzyme has approximately a 10-fold decrease in maximal activity as compared to the wild-type *B. subtilis* enzyme. The mutant enzyme also shows approximately a 140-fold and a 760-fold increase in the $[S]_{0.5}$ for aspartate and carbamoyl phosphate, respectively, as compared to the values for the wild-type *B. subtilis* enzyme. These results are consistent with the side chain of Arg-99_{BS} being important for the binding of both substrates and for catalysis. Thus, the catalytic function of this side chain is conserved between the *E. coli* and the *B. subtilis* enzymes.

Homotropic Properties of the Arg-99→Ala_{BS} and the Arg-105→Ala_{EC} Enzymes. Similar to the Arg-105→Ala_{EC} catalytic trimer, the Arg-99→Ala_{BS} enzyme exhibits sigmoidal aspartate saturation kinetics with a Hill coefficient of 1.5 (Figure 2, Table I). In order to confirm that the sigmoidal aspartate saturation kinetics are not an artifact, an activation experiment was performed. At low concentrations of cooperatively bound substrate, allosteric enzymes can be activated by competitive inhibitors of the substrate (Collins & Stark, 1971). This inhibitor activation is caused by homotropic interactions between the active sites. As seen in Figure 3, the Arg-99→Ala_{BS} enzyme is activated approximately 230% by succinate at a low concentration of aspartate and a saturating concentration of carbamoyl phosphate, while under similar conditions the *B. subtilis* wild-type enzyme is inhibited. Thus, the sigmoidal saturation kinetics are due to positive homotropic or cooperative interactions between active sites, and the enzyme has been converted into an allosteric form by the amino acid substitution.

According to the models used to explain cooperative substrate binding (Monod et al., 1965; Koshland et al., 1966), both the Arg-105→Ala_{EC} catalytic trimer and the Arg-99→Ala_{BS} enzyme are required to have at least two conformations, which differ in catalytic activity and substrate affinity. Therefore, the substitution of an alanine at position Arg-105_{EC} or Arg-99_{BS} has either caused a new conformational change to occur or eliminated a conformational change that normally occurs upon carbamoyl phosphate binding. In either case, the mutant trimer–carbamoyl phosphate complexes exist in a lower-affinity, lower-activity conformation, which is converted

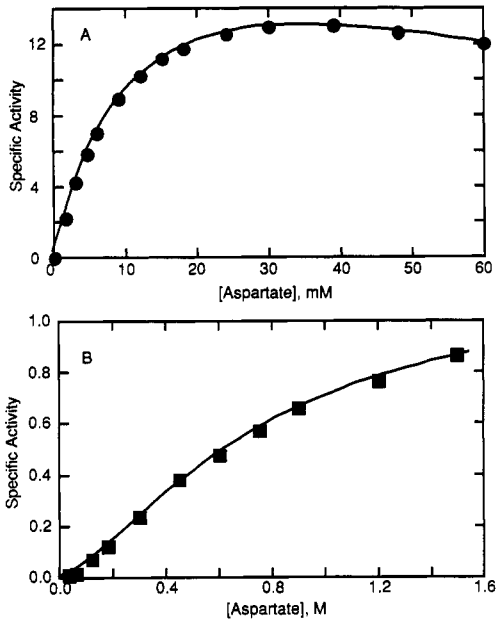

FIGURE 2: Aspartate saturation curves of the wild-type (A, ●) and Arg-99→Ala (B, ■) *B. subtilis* aspartate transcarbamoylases. Specific activity is reported in millimoles of *N*-carbamoyl-L-aspartate formed per hour per milligram of protein. Colorimetric assays were performed at 25 °C in 0.05 Tris–acetate buffer (pH 8.3) at saturating levels of carbamoyl phosphate (4.8 and 15 mM for the *B. subtilis* wild-type and Arg-99→Ala enzymes, respectively) (Pastra-Landis et al., 1981). All data points are averages of duplicates and were fit by nonlinear least squares to the Michaelis–Menten equation with a term incorporated for substrate inhibition and the Hill equation for the *B. subtilis* wild-type and Arg-99→Ala data, respectively (Pastra-Landis et al., 1978).

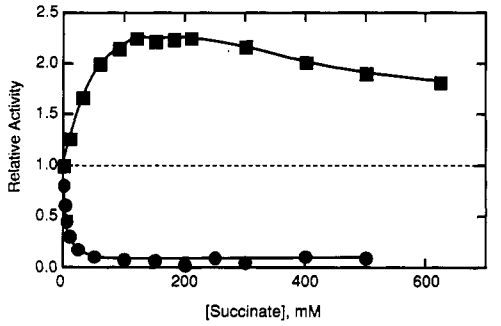

FIGURE 3: Influence of succinate on the activity of the wild-type (●) and Arg-99→Ala (■) *B. subtilis* aspartate transcarbamoylases at low aspartate concentrations (approximately 0.1 $[S]_{0.5}$ for aspartate). Colorimetric assays were performed at 25 °C in 0.05 M Tris–acetate buffer (pH 8.3) with saturating carbamoyl phosphate (4.8 and 15 mM for the *B. subtilis* wild-type and Arg-99→Ala enzymes, respectively) (Pastra-Landis et al., 1981). All data points are averages of duplicates.

to a higher-activity, higher-affinity conformation upon the binding of aspartate.

Homotropic Metabolic Regulation and Molecular Evolution. Is there a phenotypic advantage inherent to oligomeric enzymes, other than increased stability? The results presented in this work have shown that the activity of the trimeric *B. subtilis* aspartate transcarbamoylase can be transformed into a cooperative and possibly regulatory process by a single amino acid substitution. To date, single amino acid substitutions have converted two trimeric noncooperative aspartate transcarbamoylases and one trimeric noncooperative ornithine transcarbamoylase into cooperative enzymes (Kuo et al., 1989; Stebbins et al., 1989). These three enzymes show relatively low (35% and lower) amino acid sequence homology (Lerner & Switzer, 1986) and are constitutive to two different bacterial species, the progenitors of which diverged approximately 1 billion years ago (Fox et al., 1980). Thus, a small number of amino acid substitutions in an oligomeric metabolic enzyme could transform a metabolic step into a regulatory juncture. This possible evolutionary potential could explain in part the abundance of oligomeric enzymes in nature.

Registry No. Arg, 74-79-3; Ala, 56-41-7; aspartate transcarbamoylase, 9012-49-1.

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Role of Apolipoprotein E in Hepatic Lipase Catalyzed Hydrolysis of Phospholipid in High-Density Lipoproteins†

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ABSTRACT: We reported earlier that hepatic lipase (HL)-catalyzed hydrolysis of phospholipid monolayers is activated by apolipoprotein (apo) E [Thuren et al. (1991b) *J. Biol. Chem. 266*, 4853–4861]. On the basis of these studies, it was postulated that apoE-rich high-density lipoproteins (HDL) were preferred substrates for HL. In the present study, we tested this hypothesis, as well as further characterizing the activation of HL hydrolysis of phospholipid by apoE. The apoE-rich HDL, referred to as HDL-I, were isolated by heparin–Sepharose chromatography, and the phospholipid hydrolysis by HL was compared to an apoE-poor HDL, designated HDL-II. The hydrolysis of HDL-I phosphatidylcholine was approximately 3-fold higher than HDL-II, supporting the hypothesis that HL preferably hydrolyzes the phospholipids in apoE-rich HDL. In order to gain additional insight into the nature of the activation, we used phospholipid monolayers as model systems. Comparison of the ability of the two thrombolytic fragments of apoE (22 kDa, residues 1–191; 12 kDa, residues 192–299) revealed that only the 12-kDa fragment was capable of activating the hydrolysis of phospholipid by HL (1.75-fold). However, activation was less than with the intact protein (2.8-fold for apoE3), suggesting that the intact protein was required for full activation. The fact that the 12-kDa fragment, which represents a major lipid region of the protein, did activate HL suggests that activation occurs at the lipid–water interface. The three common isoforms of apoE differed significantly in their abilities to activate HL; apoE3, apoE2, and apoE4 activated HL 2.8-, 2.3-, and 2.0-fold, respectively, at a surface pressure of 12.5 mN/m. The order of affinity of the isoforms for PC monolayers (E4 > E2 > E3) was not the same as activation, suggesting that lipid binding per se was not the major factor distinguishing the abilities of the three isoforms to activate hydrolysis. Determination of the ratio of HL and apoE at a phospholipid interface revealed that the stoichiometry was approximately 1:1, suggesting that the activation of HL by apoE is the result of protein–protein interaction.

Hepatic lipase (EC 3.1.1.34) (HL)[1] hydrolyzes the *sn*-1 fatty acyl ester bonds of *sn*-3 phospholipids as well as the *sn*-1 (*sn*-3) ester bonds of mono-, di-, and triacylglycerols (Jackson, 1983; Kinnunen, 1984; Waite, 1987). The enzyme is located on the surface of vascular endothelium in liver where it catalyzes the hydrolysis of lipid components of plasma lipoproteins (Kinnunen, 1984; Waite, 1987). The role of HL in lipoprotein metabolism is unclear. Its broad substrate specificity and the fact that this enzyme does not absolutely require an apolipoprotein (apo) cofactor for activity have resulted in uncertainty as to what the physiological substrate(s) for this enzyme is (are). HL has been proposed to hydrolyze intermediate-density lipoprotein triacylglycerol and chylomicron remnant triacylglycerol (Dolphin, 1985; Breslow, 1988; Carlson et al., 1986; Little & Connelly, 1986; Breckenridge et al., 1982). Also it has been suggested that HL hydrolyzes HDL-II and/or apoE-rich HDL-I phospholipid (Kinnunen, 1984; Belcher et al., 1985; Jansen et al., 1980; Van Tol et al., 1980; Bamberger et al., 1985; Kuusi et al., 1979) and that the hydrolysis of HDL phospholipids by HL enhances cholesterol (ester) uptake by liver cells (Van't Hoof et al., 1981).

The effect of apolipoproteins on HL activity varies greatly depending on the assay system (Kinnunen & Ehnholm, 1976; Shinomiya, 1982; Kubo et al., 1982; Jahn et al., 1981, 1983; Landis et al., 1987). This is because the activity of HL as well as other lipolytic enzymes is greatly affected by the physicochemical state of the substrate (Waite, 1987). Using a well-controlled monolayer technique with a zero-order trough

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[1] Abbreviations: apo, apolipoprotein; apoE3-12 kDa, 12-kDa thrombolytic fragment of apoE3/E3 isoform; apoE3-22 kDa, 22-kDa thrombolytic fragment of apoE3/E3 isoform; bME, β-mercaptoethanol; diC12PE, 1,2-didodecanoylphosphatidylethanolamine; HDL, high-density lipoprotein(s); PC, phosphatidylcholine; PE, phosphatidylethanolamine; HL, hepatic lipase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

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