Reaction Selectivity of *Burkholderia cepacia* (PS-30) Lipase as Influenced by Monoacylation of *sn*-Glycerol

Xun Fu and Kirk L. Parkin*

University of Wisconsin, Department of Food Science, Babcock Hall, Madison, Wisconsin 53706

ABSTRACT: Reaction selectivities were determined in multi-competitive reactions mediated by *Burkholderia cepacia* lipase (Amano PS-30) at a water activity of 0.19 in hexane. Saturated FA (C4–C18 even chain) and oleic acid (C18:1) were reacted with a single alcohol, glycerol, or α- or β-MAG, containing C4, C10, C16, or C18:1 individually as alcohol cosubstrate. Similar ordinal patterns of FA selectivity, with C8, C10, and C16 preferred over others, were generally observed for incorporation of FA into specific acylglycerol (AG) pools of the 24 specific cases evaluated. The three exceptions were enrichment of C14 and C18 in the MAG pool with α-C16-MAG substrate, and a general suppression of >C8 incorporation into the TAG pool for reactions with α-C10- and α-C16-MAG. PS-30 lipase selectivity toward MAG was in descending order: α/β-C4-MAG > β-C10-MAG > β-C16-MAG > α/β-C18:1-MAG > α-C10-MAG > α-C16-MAG. Selectivity in channeling CX of the original CX-MAG substrates into higher AG species was in descending order: α-C10-MAG ~ α-C16-MAG > α-C18:1-MAG > β-C10-MAG ~ β-C16-MAG ~ β-C18:1-MAG > α/β-C4-MAG. Generally, MAG were better acyl donors than FA for esterification reactions leading to DAG formation. These observations are relevant to the design of biocatalytic processes intended to yield specifically structured TAG.

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KEY WORDS: *Burkholderia cepacia,* esterification, fatty acid, lipase, monoacylglycerol, selectivity.

A focus of over a decade of recent research on exploiting the synthetic power of lipases in organic media is the prospect of preparing value-added or functional "structured glycerides" for food and pharmaceutical industries (1). Examples of lipids modified by lipase to confer additional value and/or functionality include low-calorie fats (2), cocoa butter substitutes (3), and nutritional lipids (4,5). The applications-driven research in this area has focused largely on using specific lipid mixtures or even pure reaction components to prepare specific targets of synthesis. Much of this body of literature has been rather empirical and sparingly reliant on features of lipase selectivity, such as regioselectivity. Indeed, the commercial and economic justifications for using a lipase over any chemical modification process are embedded in the multifaceted patterns of selectivity offered by an enzymatic reaction.

Present address of first author: University of Wisconsin, School of Pharmacy, Rennebohm Hall, 777 Highland Ave., Madison, WI 53705.

*To whom correspondence should be addressed at University of Wisconsin, Department of Food Science, Babcock Hall, 1605 Linden Dr., Madison, WI 53706. E-mail: klparkin@wisc.edu

To facilitate the broadest possible application of lipases to modify lipids for added value, it is imperative to develop an understanding of lipase selectivity patterns in a manner that allows the application of such knowledge to a host of possible reactions, where the identity and levels of lipid substrates may be quite varied. One approach that fulfills this need is the determination of relative kinetic constants that define the substrate selectivity patterns of various lipases. Selectivity constants may be used to predict the pattern of lipase reactivity under a broad range of conditions where substrate identity and levels are variable, since kinetic constants are not concentration-dependent. Many groups have used this approach to quantify the discriminatory power of various lipases among substrates for esterification reactions between FA and alcohols (6–10).

Our approach has focused on kinetically characterizing lipase-mediated reactions that model the progressive esterification of glycerol to yield acylglycerols (AG) leading to TAG assembly (9,10). Reaction selectivity among a broad range of FA for selected lipases in reactions with (iso)propanol and propanediols (to simulate reactivity of di- and mono-AG species, respectively) and glycerol has been reported. These studies led us to believe that FA selectivity of a lipase may be altered by the nature of the alcohol cosubstrate, as esterification reactions progress through the incremental steps from acylation of glycerol to yield TAG. Indeed, our preliminary report suggested that lipase FA selectivity in esterification reactions with monomyristoylglycerol was different from that with glycerol (11). The implications of this finding are that reaction selectivity for the complete process of assembling TAG from glycerol and FA may be quite complicated and varied for each step of assembly, making reaction design to yield a target TAG quite challenging. We were unable to find any prior literature account of the impact that progressive acylation of the glycerol backbone may have on FA selectivity of lipase for subsequent acylation steps. Accordingly, the objective of this study was to determine whether FA selectivity in esterification reactions mediated by *Burkholderia cepacia* lipase is different in reactions with glycerol compared with MAG, the latter as influenced by acyl chain length and site of *sn*-glycerol attachment.

MATERIALS AND METHODS

Materials. The lipase from *Burkholderia* (formerly *Pseudomonas*) *cepacia* was obtained as Lipase PS-30 from Amano

Enzyme USA (Lombard, IL). The enzyme was immobilized on Celite (1:5, w/w) according to common procedures (9). *sn*-Glycerol-1(3)-monobutyrin (α-C4-MAG), *sn*-glycerol-1(3)-monocaprin (α-C10-MAG), *sn*-glycerol-1(3)-monopalmitin (α-C16-MAG), and *sn*-glycerol-1(3)-monoolein (α-C18:1 MAG) were purchased from Doosan Serdary Research Laboratories (Toronto, Canada). All other reagents were obtained from Fisher Scientific (Chicago, IL), Aldrich Inc. (Milwaukee, WI), or Sigma Chemical Co. (St. Louis, MO).

Preparation of sn*-2-MAG. sn*-Glycerol-2-monobutyrin (β-C4-MAG), *sn*-glycerol-2-monocaprin (β-C10-MAG), *sn*-glycerol-2-monopalmitin (β-C16-MAG), and *sn*-glycerol-2-monoolein (β-C18:1-MAG) were prepared by alcoholysis reactions with the corresponding monoacid TAG based on established methods (12,13). A mixture of TAG (0.01 mol), Celite-immobilized PS-30 (400 mg), ethanol (0.12 mol), and *tert*-butyl methyl ether (*t*BME) (40 mL) was orbitally shaken at 300 rpm and 40°C for 12 h. The reaction mixture was centrifuged at 850 × *g* to remove enzyme, and the ethereal layer was concentrated. The product mixture was separated by preparative TLC (PK6F silica gel 60Å, 1000 μm; Whatman, Clifton, NJ) using a developing solvent of hexane/diethyl ether (2:3, vol/vol) to afford the corresponding β-MAG species at yields of 18–25%. Structure was confirmed by ^{1}H NMR (model AM-300 NMR spectrometer; Bruker Instruments), which revealed two signals representative of α-H and β-H in the range of 3.6–5.0 ppm (e.g., 3.78 and 4.9 ppm for β-C10-MAG).

Competitive enzyme reactions. The reaction system routinely consisted of 50 mM of a single CX-MAG species (α- or β-configured C4-, C10-, C16-, or C18:1-MAG) as alcohol cosubstrate, and 80 mM each of multiple FA (designated as saturated C4–C18 FA and unsaturated C18:1 FA). The FA representing the acyl group comprising the specific MAG cosubstrate was not included in the group of FA reactants, making the total FA concentration of 640 mM. Glycerol was also employed as a cosubstrate instead of MAG for comparison; glycerol was immobilized on silica gel (grade 9385, 230–400 mesh 60Å; Merck, Darmstadt, Germany) according to reported methods (14) to minimize multiphase behavior otherwise caused by free glycerol. Reactions were carried out in *n*-hexane (20 mL), and the water activity (a_w) was controlled by the salt hydrate pair (1.2 g each) of anhydrous Na_2HPO_4 and $Na_2HPO_4{\cdot}(H_2O)_2$ to poise a_w at 0.19 (15). MAG were used at near their solubility limit in hexane (16), but there was no visual indication (turbidity) that MAG were insoluble under the conditions used.

Sampling and analysis. Reaction mixtures were preincubated at 35°C for 30 min, and Celite-bound enzyme (100 mg) was added to initiate the reaction. Subsamples of 0.35 mL were withdrawn from the reaction mixture at intervals of ~30 min and centrifuged at 850 × *g* to remove particulate matter (enzyme and salt). The clarified sample solution (0.15 mL × 2) was then applied to analytical TLC plates (LK6F silica gel 60Å, 250 μm; Whatman), and the plate was developed in a solvent system of petroleum ether/diethyl ether/acetic acid (65:35:1, by vol). The silica gel zones corresponding to separated bands of MAG, DAG, and TAG were scraped and transferred to a 10-mL screw-capped tube.

Derivatization of AG products (MAG, DAG, and TAG) to yield FAME was performed by adding 0.5 M methanolic sodium methoxide (2 mL) and rotary shaking the mixture in test tubes at 50°C for 30 min (17). Hexane (0.5 mL) containing limonene (0.1 μL) as internal standard and saturated sodium chloride solution (4 mL) were then added to the tube. The resulting mixture was vortexed for 3 min and centrifuged at 850 × *g* in a clinical centrifuge for 5 min. Finally, the supernatant containing FAME was analyzed by a Hewlett-Packard 6890 series gas chromatograph equipped with an FID and HP-INNOWAX cross-linked PEG capillary column (30 m × 0.32 mm × 0.5 μm). The oven temperature program was ramped from 50°C, initially held for 2 min, to 220°C at 18°C/min and from 220 to 250°C at 10°C/min and finally held at 250°C for 4 min. The injector, in splitless mode, and detector temperature were set at 220 and 230°C, respectively. Individual FAME peaks were quantified by external standards curves and by using the internal limonene standard.

FA selectivity and kinetic analysis. The competitive factor (also commonly referred to as relative selectivity constant or α-value) among multiple FA substrates was determined based on the kinetic model advanced previously (6,18) as applied in previous studies (8–10). α-Values were analyzed experimentally as progress of reactivity of one (or more) substrate(s) relative to a reference substrate (C8 FA in this study), according to the following equation:

$$\log ([C_o]_A/[C_o - C_x]_A) = \alpha \log ([C_o]_B/[C_o - C_x]_B) \qquad [1]$$

where subscripts *A* and *B* represent competing substrates and the reference substrate, respectively. $[C_o]$ is the initial substrate concentration and $[C_x]$ is the corresponding product concentration; hence, $[C_o - C_x]$ represents the substrate concentration remaining at any given time interval, *x*. The slope of the log–log plot of the equation is the α-value for any pair of FA substrates. Since the α-value of the reference substrate (C8 FA) is taken as 1.0, a competing substrate with a greater α-value is by comparison more reactive (selective) with the lipase than the reference FA substrate (C8).

Initial reaction rates (v_o) were also calculated from progress curves representing total FA esterified into the AG pool (MAG + DAG + TAG). This was done by estimating initial (linear) velocities, except in one case (α-C10-MAG) where a curvilinear progress curve (lag followed by accelerating rate) occurred and an average reaction velocity over the entire reaction period was estimated instead. This was done to provide an assessment and comparison of lipase reaction selectivity among different MAG species. It is recognized that as reactions progress, the original CX-MAG pool will become "contaminated" by an increasing level of non-CX-MAG species. Although this may compromise some of the integrity of this analysis, it can be argued that using "initial reaction rate data" serves to minimize the impact of this

phenomenon, and the general conclusion reached as to relative selectivity of the lipase for CX-MAG species based on v_o estimates is valid.

Analysis of FA selectivity and overall esterification reaction rate was also done for reaction mixtures employing the full set of FA (C4–C18, C18:1; 720 mM FA total) with 50 mM of glycerol for comparison. Because glycerol was immobilized on silica gel, we recognize that there are limits to making comparisons with reaction rates employing CX-MAG species for the purposes of establishing enzyme selectivity. However, reaction progress curves with glycerol are illustrated in the Figures describing reactions with CX-MAG species for context and benchmarking purposes. Since the role of silica gel is to provide a reservoir to mete out and maintain saturating levels of free glycerol in the solvent as it is consumed by reaction (14), no impact on FA selectivity is expected from the use of silica gel (10,14).

In many cases, kinetic analysis could be applied to the incorporation of CX originating from CX-MAG into DAG and TAG pools, and α-values were determined for these reaction steps. In other cases, the progress of CX incorporation from CX-MAG into DAG and TAG pools did not yield linear log–log plots, and estimates of α-values could not be provided (as indicated in the figures).

RESULTS AND DISCUSSION

Reaction design considerations. The primary consideration in the design of these experiments was to create reaction conditions that were kinetically favorable to net esterification and to minimize the reverse hydrolytic process, especially for the step involving acylation of preformed MAG to the corresponding DAG. Based on mass action and reaction equilibria, the forward esterification reaction is favored by elevating both FA and MAG concentration, whereas the reverse and undesired hydrolytic reaction is also favored by elevating the MAG concentration. Thus, our strategy involved using elevated total FA concentrations at 640 mM together with a limited MAG concentration [50 mM instead of 175 mM used previously (11)] as being most conducive to providing a kinetic analysis of esterification reaction selectivity. An a_w of 0.19 was also chosen to favor esterification reactions. This condition was judged from preliminary studies to meet the needs of having sufficient levels of MAG, DAG, and TAG to obtain sensitive and accurate analyses. Despite the intent of these reaction design efforts, experimental data provided evidence that an initial phase of CX-MAG hydrolysis occurred to some degree within the first sampling interval. This may reflect a rapid initial adjustment of multiple reaction equilibria in the system immediately following the combining of enzyme and substrates.

Reactions with C4-MAG species. Esterification reactions by PS-30 lipase were up to twice as fast (based on linearized/initial rates of non-C4 FA incorporation) with either α/β-C4-MAG species as alcohol cosubstrate compared with glycerol (Figs. 1A, C; Table 1). Non-C4 FA were incorporated into DAG and TAG species faster with β-C4-MAG than α-C4-MAG as alcohol cosubstrate. As reactions were allowed

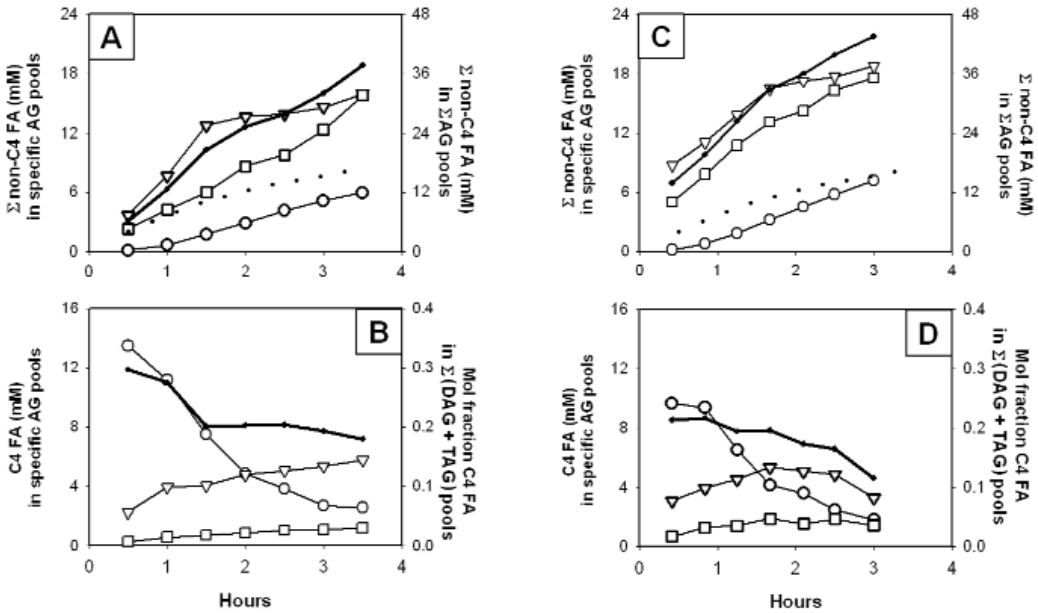

FIG. 1. Progress of esterification reactions between FA and C4-MAG. Incorporation of non-C4 FA into specific acylglycerol (AG) pools (open symbol plots, left ordinate) and total non-C4 FA esterified in all AG pools (bold line plots, right ordinate; reaction with glycerol shown as dotted line) is shown with α-C4-MAG (panel A) and β-C4-MAG (panel C) as substrate. Recovery of esterified C4 from original C4-MAG in specific AG pools (open symbol plots, left ordinate) and in combined DAG + TAG pools (bold line plots, right ordinate) is shown with α-C4-MAG (panel B) and β-C4-MAG (panel D) as substrate. Results are representative of two experiments that showed identical trends: ○, MAG; ▽, DAG; □, TAG; –●–, ΣAG.

TABLE 1
Profiles of Esterified Acylglycerol (AG) Reaction Components at Initial and Final Sampling Intervals

Initial alcohol cosubstrate (CX-MAG)		Initial reaction velocity (v_o)		Esterified components (mM) at initial sampling interval			Esterified components (mM) at final sampling interval		
		v_o (mM/h)	v_o, MAG : v_o, glycerol	Non-CX FA	CX	ΣAG[a]	Non-CX FA	CX	ΣAG[a]
Glycerol		6.7	1.0	4.04[b]	—	—	16.7[b]	—	—
C4-MAG	(α)	13	1.9	6.17	16.0	17.5 *0.78, 0.17, 0.05*	37.8	9.52	25.1 *0.34, 0.43, 0.23*
	(β)	15	2.2	13.9	13.4	17.6 *0.56, 0.33, 0.11*	43.6	6.53	26.4 *0.34, 0.42, 0.24*
C10-MAG	(α)	2.8[c]	0.41	0.46	29.6	29.5 *0.98, 0.02, 0.00*	9.16	23.7	23.8 *0.67, 0.28, 0.05*
	(β)	11	1.7	4.48	36.3	35.1 *0.86, 0.12, 0.02*	31.1	12.1	24.4 *0.43, 0.38, 0.20*
C16-MAG	(α)	0.65	0.10	0.63	12.5	12.8 *0.97, 0.02, 0.01*	2.63	28.0	28.0 *0.91, 0.08, 0.01*
	(β)	9.1	1.4	8.18	31.0	30.0 *0.74, 0.20, 0.05*	27.9	10.5	20.7 *0.41, 0.34, 0.25*
C18:1-MAG	(α)	6.5	0.97	1.62	25.8	26.0 *0.95, 0.04, 0.00*	20.8	14.6	20.8 0.47, 0.40, 0.12
	(β)	7.4	1.1	5.89	28.6	29.7 *0.86, 0.12, 0.02*	24.8	14.4	22.5 *0.45, 0.35, 0.20*

[a]ΣAG was calculated as Σ {(mM FA in MAG) + [(mM FA in DAG)/2] + [(FA in TAG)/3]}, from combined "CX FA" and "non-CX FA" species. Numbers in italics represent mol fraction of accumulated AG species as MAG, DAG, TAG.
[b]Reaction mixtures with glycerol employed all FA within the series of C4–C18, C18:1. There is no "non-CX" or "CX" FA species in this case.
[c]Reaction velocity was calculated as an average velocity for the entire reaction period and does not represent a true v_o.

to progress, C4 became diminished in the MAG and enriched in the DAG and then TAG pools, as expected from the progressive esterification of FA to α/β-C4-MAG (Figs. 1B, D).

A somewhat surprising result was that after an initial lag period, non-C4 FA substrates started to accumulate in the MAG pool (Figs. 1A, C). (Analogous observations were made for all other reaction systems evaluated.) This event can occur principally through (i) C4-group removal from a mixed-acid C4-containing DAG species or (ii) C4-group removal from α/β-C4-MAG followed by esterification of the resulting glycerol with a non-C4 FA, none of which were the intended reaction pathways to be examined in this study. A direct assessment of these possibilities was not conducted, and both routes are kinetically similar in that they require two catalytic events, hydrolysis and esterification. However, based on anticipated mass action effects, the data appear more supportive of the prospect of hydrolysis of α/β-C4-MAG followed by esterification being the major route of non-C4-MAG accumulation. As of the first sampling interval, of the original 50 mM α/β-C4-MAG, about 32–33 mM free glycerol was calculated to be present (Table 1; Fig. 1), and this was consistent with the ~13–16 mM C4 remaining esterified in the ΣAG pools (Table 1; Figs. 1B, D). Since DAG accumulated only to about 3–6 mM as of the first sampling interval, it is difficult to imagine DAG species as the primary source of the calculated 34–37 mM C4 de-esterified in the reaction mixture at this time. Also, the a_w of 0.19 used in this study favors esterification, and even if there is an initial thermodynamic adjustment toward net hydrolysis, the dominant AG species in the system initially (50 mM MAG) would likely serve as the primary source of evolved free glycerol and C4.

The levels of the C4 mol fraction in combined DAG and TAG pools generally declined from 0.2–0.3 to 0.1–0.2 throughout the reaction period, less than the values expected (0.50 for DAG, and 0.33 for TAG) if C4-MAG species were entirely and directly channeled through subsequent esterification steps with non-C4 FA (Figs. 1B, D). These data are evidence of exclusion of C4 from these two AG pools, reflecting product selectivity of the reaction.

PS-30 lipase selectivity for incorporation of non-C4 FA into the discrete AG pools for reactions with α/β-C4-MAG exhibited a preference for FA C8, C10, and C16 in all cases (Fig. 2), and the ordinal patterns of FA selectivity were similar to esterification reactions with glycerol as alcohol cosubstrate (diamond insets on Figs. 2A–C). The incorporation of C4 into higher glycerides (DAG and TAG) yielded large relative selectivity constants (α-values), appearing as black bars in Figure 2 (panels B–F). The large α-values observed for C4 incorporation into the DAG pool were likely conferred only through the channeling of C4-MAG into the DAG pool through progressive esterification, since the PS-30 lipase was discriminatory against C4 FA in direct (inter)esterification reactions (Figs. 2A–C, diamond insets).

The α-values for C4 compared with other FA for incorporation into higher AG pools are not very meaningful for a given reaction step leading to a specific AG pool. However, how the α-values for C4 change as one proceeds from the DAG to the TAG pool (Figs. 2B, E, respectively, going to Figs. 2C, F), and how these values and trends compare with

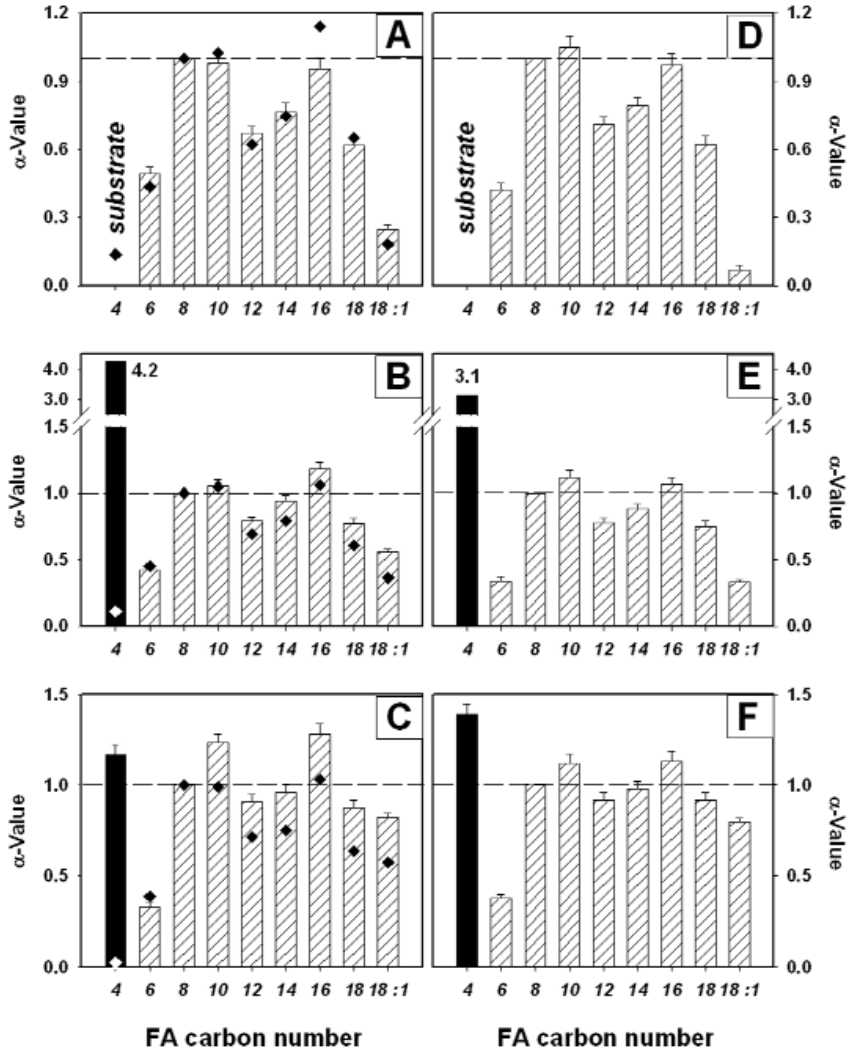

FIG. 2. Relative selectivity constants (α-values) with C4-MAG substrates for incorporation of FA into specific AG pools. Reactions with α-C4-MAG are shown for incorporation of FA into MAG (panel A), DAG (panel B), and TAG (panel C) pools. Reactions with β-C4-MAG are shown for incorporation of FA into MAG (panel D), DAG (panel E), and TAG (panel F) pools. Corresponding α-values for reactions between FA and glycerol are shown as diamond insets (in only panels A–C). Estimating the selectivity of incorporation of C4 from original C4-MAG into the MAG pool was considered inappropriate and therefore designated as "substrate" (panels A and D); where data fit the kinetic model for analysis, incorporation of C4 into DAG (panels B and E) and TAG (panels C and F) pools was estimated and appears as black bars. Results are expressed as means ± range of observations for two experiments. For abbreviation see Figure 1.

those of other FA used as the original α/β-FA-MAG substrates can be revealing. In the present case, the rather small α-value for C4 entering the DAG pool and the even smaller α-value for C4 entering the TAG pool reflect PS-30 lipase being discriminatory against C4 entering higher AG pools, corroborating an observation made earlier for mol-fraction analysis (Figs. 1B, D). This conclusion will become more evident as results are presented for reactions with other α/β-FA-MAG.

Reactions with C10-MAG species. Relative to reaction rates with glycerol as alcohol cosubstrate, esterification reactions by PS-30 lipase were ~60% slower with α-C10-MAG (Fig. 3A) and about 70% faster (based on initial rates of non-C10 FA incorporation) with β-C10-MAG (Fig. 3C) as alcohol cosubstrate (Table 1). The faster rate of incorporation of non-C10 FA into DAG and TAG species with β-C4-MAG compared with α-C4-MAG was associated with an accelerated decline of C10-MAG in the former case (Figs. 3B, D).

As reactions were allowed to progress, C10 became diminished in the MAG and enriched in the DAG and then TAG pools, as expected from progressive esterification of FA to α/β-C10-MAG (Figs. 3B, D). Recovery of C10 esterified in the ΣAG pools at the initial sampling interval was 30–36 mM of the initial 50 mM of C10 esterified as α/β-MAG, indicat-

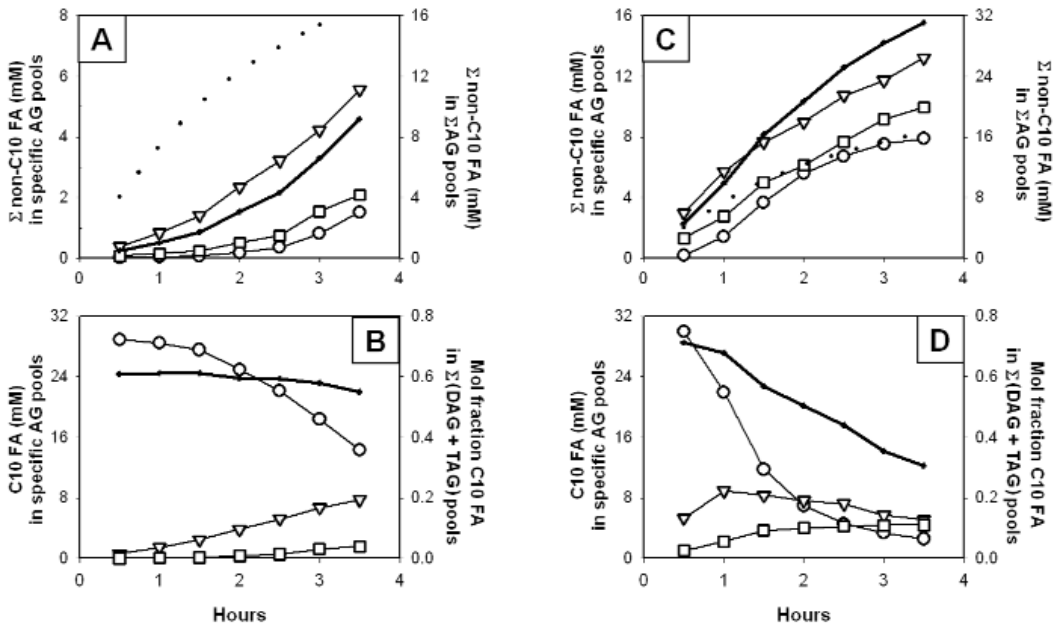

FIG. 3. Progress of esterification reactions between FA and C10-MAG. Figure legend is the same as for Figure 1 except the incorporation of non-C10 FA into AG appears in panel A for α-C10-MAG and panel C for β-C10-MAG as substrates, and the recovery of esterified C10 from original C10-MAG is shown in panel B with α-C10-MAG and panel D with β-C10-MAG as substrates. For abbreviation see Figure 1.

ing that limited net hydrolysis of C10 occurred (Fig. 3), relative to C4 from α/β-C4-MAG (*cf.* Fig. 1; Table 1). The mol fraction of C10 in combined Σ(DAG + TAG) pools was maintained at 0.5–0.6, and continually declined from 0.7 to 0.3 for the reaction period for reaction systems based on α-C10-MAG and β-C10-MAG cosubstrates, respectively (Figs. 3B, D). Relative to the observations made with C4 originating from C4-MAG substrates (Figs. 1B, D), C10 remained selectively enriched in the accumulated Σ(DAG + TAG) pools.

An obvious lag period was observed for the incorporation of non-C10 FA substrates into various accumulating AG pools when α-C10-MAG was the cosubstrate (Fig. 3A), precluding a true estimate of v_o (Table 1). Based also on the premise advanced earlier that non-CX FA become esterified as accumulating MAG only after CX-MAG hydrolysis to yield free glycerol, it seems evident that α-C10-MAG reactivity was attenuated for both hydrolytic and esterification processes. This may be due to a steric constraint on reactivity (nonproductive binding) and/or the enzyme exhibiting selectivity for the ground state of α-C10-MAG (tight binding, limited reaction).

PS-30 lipase selectivity for non-C10 FA incorporation into the discrete AG pools for reactions with α/β-C10-MAG exhibited a preference for FA C8 and C16 in all cases (Fig. 4). The ordinal patterns of FA selectivity in esterification reactions with α/β-C10-MAG were similar to those recorded for esterification reactions with glycerol as alcohol cosubstrate (diamond insets on Figs. 4A–C). However, it appeared that relative enzyme selectivity for C8 FA was enhanced over other FA for esterification reactions yielding TAG from the original α-C10-MAG substrate (Fig. 4C).

The incorporation of C10 into higher glycerides (DAG and TAG) could be kinetically characterized by relative selectivity constants (α-values) in two cases (Figs. 4B, F). The large α-values observed for C10 incorporation into the DAG pool is likely conferred by simple channeling of α-C10-MAG into the DAG pool *via* (inter)esterification, coupled with the preference of PS-30 lipase for this FA (liberated by prior α-C10-MAG hydrolysis) as indicated by the diamond insets in Figure 4. With regard to reaction product selectivity, PS-30 lipase was more selective for incorporating C10 than C4 into higher AG pools from the respective C4/C10-α/β-MAG substrates, based on greater α-values for C10 (Figs. 4B, F) over those for C4 (Figs. 2B, C, E, F).

Reactions with C16-MAG species. Relative to reaction rates with glycerol as alcohol cosubstrate, esterification reactions by PS-30 lipase were only 10% as fast with α-C16-MAG (Fig. 5A) and about 50% faster (based on initial rates of non-C16 FA incorporation) with β-C16-MAG (Fig. 5C) as alcohol cosubstrate (Table 1). Non-C16 FA were readily incorporated into DAG and TAG species with β-C16-MAG as original cosubstrate (Fig. 5C), but only into DAG with α-C16-MAG as cosubstrate (Fig. 5A). The lack of accumulation of non-C16 FA in both TAG and MAG pools in the case of reactions with α-C16-MAG apparently reflects a lack of reactivity toward further esterification of C16-containing DAG species to yield TAG, and a lack of net hydrolysis of α-C16-MAG to limit the supply of free glycerol for esterification reactions with non-C16 FA to yield MAG. However, at the initial sampling interval, only 12–13 mM each (of the original 50 mM) of esterified C16 and glycerol were recovered in the ΣAG pools in the α-C16-MAG-based reaction mixture (Figs. 5A, B; Table 1). The

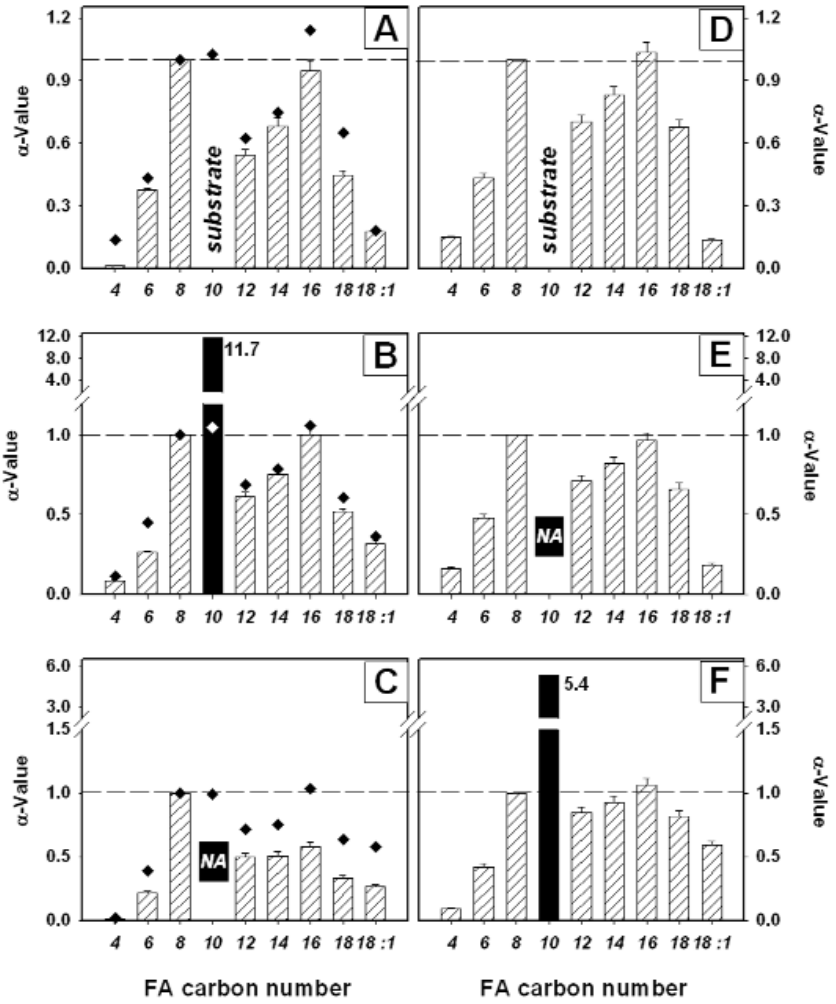

FIG. 4. Relative selectivity constants (α-values) with C10-MAG substrates for incorporation of FA into specific AG pools. Figure legend is the same as for Figure 2 except that reactions with α-C10-MAG are shown for incorporation of FA into MAG (panel A), DAG (panel B), and TAG (panel C) pools, and reactions with β-C10-MAG are shown for incorporation of FA into MAG (panel D), DAG (panel E), and TAG (panel F) pools. "NA" indicates that data for C10 from original C10-MAG substrate did not fit the kinetic model to allow estimation. For abbreviation see Figure 1.

calculated and corresponding 37 mM free glycerol should have otherwise permitted facile incorporation of non-C16 FA into the accumulating MAG pool. Thus, we conclude that reactivity of α-C16-MAG suffered from a restricted availability for reaction. This may reflect enzyme selectivity but may also reflect physical phenomena unique to α-C16-MAG. The reaction conditions used were at or near the solidification point and/or level of saturation (16,19), and if aggregates of substrates are formed, this may have a profound influence on reaction rates and selectivity (20).

The mol fraction of C16 in the Σ(DAG + TAG) pools was maintained at 0.5–0.6, and continually declined from 0.5 to 0.3 for the entire reaction period for reaction systems based on α-C16-MAG and β-C16-MAG cosubstrates, respectively (Figs. 5B, D). These trends are consistent with the relative enrichment of C16 expected where only DAG accumulated in the α-C16-MAG reaction system (Fig. 5A), and where both DAG and TAG accumulated in the β-C16-MAG reaction system (Fig. 5C).

PS-30 lipase selectivity for incorporation of non-C16 FA into the discrete AG pools for reactions with α/β-C16-MAG generally exhibited a preference for FA C8 and C10 and reflected the ordinal patterns of FA selectivity characteristic of esterification reactions with free glycerol (Fig. 6). However, there were two notable exceptions, both with the α-C16-MAG reaction system. One was the enhanced selectivity toward C14 and C18 incorporation into the MAG pool (Fig. 6A), and this altered selectivity appeared only with α-C16-MAG among all the α/β-CX-MAG species tested. The only explanation we can offer is that esterification reactions with α-C16-MAG were indeed enhanced in selectivity for C14 and C18 FA, and the accumulation of C14- and C18-MAG species resulted from hydrolysis of the corresponding C16, CX-DAG species. We suggested earlier that DAG hydrolysis is the less

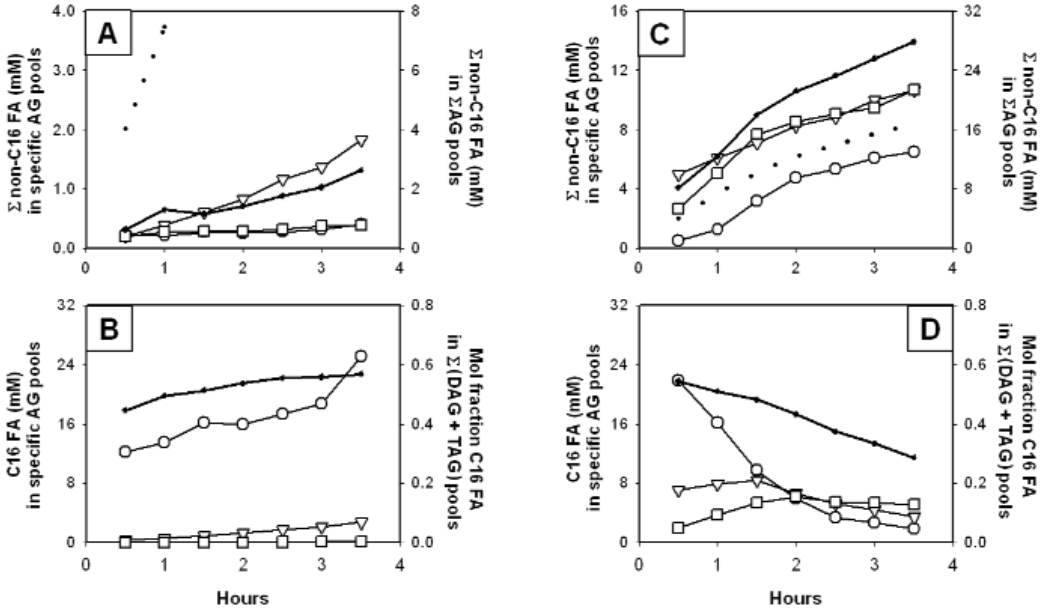

FIG. 5. Progress of esterification reactions between FA and C16-MAG. Figure legend is the same as for Figure 1 except the incorporation of non-C16 FA into AG appears in panel A for α-C16-MAG and panel C for β-C16-MAG as substrates, and the recovery of esterified C16 from original C16-MAG is shown in panel B with α-C16-MAG, and panel D with β-C16-MAG as substrates. For abbreviation see Figure 1.

likely route to MAG accumulation (bearing non-CX FA from CX-MAG substrates) than esterification of glycerol (resulting after initial CX-MAG hydrolysis). However, esterification of the original CX-MAG substrate to yield DAG followed by hydrolysis remains a viable, if secondary, route to yield non-CX-MAG. Furthermore, in this specific case, non-C16-MAG accumulated to only a limited extent (Fig. 5A) relative to reactions with other MAG species, implying that the major route of non-CX-MAG accumulation suggested in other cases was of diminished importance in this specific case. We note that our similar reaction systems employing the closely related α-C14-MAG species yielded enhanced selectivity toward C16 and C18 with this enzyme (11). Subsequent studies with appropriate DAG species would help verify the basis for this enhanced selectivity. The other exception was the specific enhancement in relative enzyme selectivity for C8 FA incorporation into TAG (Fig. 6C), a trend also noted earlier for reactions with α-C10-MAG (Fig. 4C).

Where they could be estimated, the large α-values observed for C16 incorporation into the DAG and TAG pools were likely caused by the channeling of C16-MAG into these pools through progressive esterification reactions, coupled with the general selectivity of the lipase for C16 FA (accumulating from any C16-MAG hydrolysis).

Reactions with C18:1-MAG species. Esterification reactions by PS-30 lipase were about as fast with α-C18:1-MAG (Fig. 7A) or slightly faster with β-C18:1-MAG (Fig. 7C) as analogous reactions with free glycerol, based on initial rates of non-C18:1 FA incorporation into all AG pools (Table 1). There was a longer lag period prior to accumulation of MAG and especially TAG in the case of α-C18:1-MAG, consistent with the faster reaction rate with β-C18:1-MAG. As reactions were allowed to progress, C18:1 became diminished in the MAG and enriched in the DAG and then TAG pools, as would be expected from the progressive esterification of FA to α/β-C18:1-MAG (Figs. 7B, D). At the first sampling interval, 26–30 mM each of C18:1 FA and free (calculated) glycerol were recovered esterified in ΣAG pools, indicating that <50% net hydrolysis of α/β-C18:1-MAG occurred (Table 1). The levels and trends in the mol fraction of C18:1 in the combined DAG and TAG pools generally declined from 0.4–0.5 toward 0.3 during the course of reactions, with greater enrichment of 18:1 maintained in reactions employing α-C18:1-MAG as substrate.

PS-30 lipase selectivity for incorporation of non-C18:1 FA into the discrete AG pools for reactions with α/β-C18:1-MAG exhibited a preference for FA C8, C10, and C16 in all cases (Fig. 8), and the ordinal patterns of FA selectivity were similar to esterification reactions with glycerol as alcohol co-substrate (diamond insets on Figs. 8A–C). The preference (large α-values) for incorporation of C18:1 into higher glycerides (DAG and TAG), appearing as black bars in Figure 8 (panels B, C, E, F), is likely conferred primarily through the channeling of C18:1-MAG into these AG pools by progressive esterification, as the PS-30 lipase is not very reactive with C18:1 FA in reactions with free glycerol as substrate (Figs. 8A–C) to contribute much to this behavior.

General discussion. The overall objective of this work was to gain insight into the patterns of reaction selectivity of PS-30 lipase in terms of successive steps in assembling TAG from MAG or glycerol and FA. Selectivity can be assessed using several criteria, which provide the basis for the ensuing

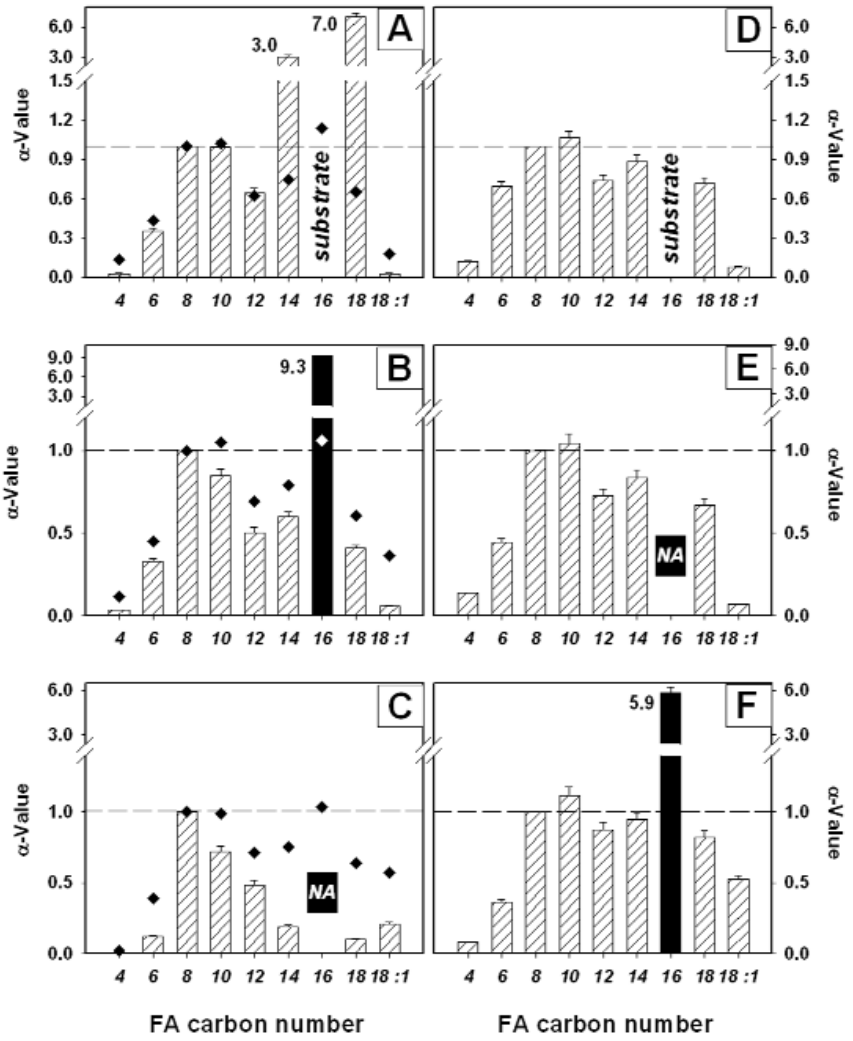

FIG. 6. Relative selectivity constants (α-values) with C16-MAG substrates for incorporation of FA into specific AG pools. Figure legend is the same as for Figure 2 except that reactions with α-C16-MAG are shown for incorporation of FA into MAG (panel A), DAG (panel B), and TAG (panel C) pools, and reactions with β-C16-MAG are shown for incorporation of FA into MAG (panel D), DAG (panel E), and TAG (panel F) pools. For abbreviation see Figure 1.

discussion. The observed selectivity in the reaction systems studied is a reflection not only of intrinsic lipase selectivity but also of how this is modulated by the specific choices of solvent, salt hydrate, and a_w that were made. In addition, reactant partitioning and interfacial activity of components may influence the results obtained, and these properties may change as reactions progress and reactants are transformed. Our determinations of reaction selectivity are based on net flow of FA into accumulating AG pools originating from a specific MAG species, and even though net esterification of FA occurred, this process reflects the composite processes of esterification, interesterification, and hydrolysis. It is recognized that any role acyl migration may have in reaction selectivity remains embedded in the results presented, as no attempt was made to account for these factors. Consideration of the facts that the time frame of our analyses was restricted to 3.5 h and that acyl migration rates for β-MAG and α,β-DAG are on the order of <5%/h in hexane (21,22) suggests that this factor had a minor impact on the results.

On the basis of initial reaction rates (Table 1), PS-30 selectivity toward MAG species was in the following descending order: α/β-C4-MAG > β-C10-MAG > β-C16-MAG > α/β-C18:1-MAG (~ glycerol) > α-C10-MAG > α-C16-MAG. This pattern is consistent with the known *sn*-1,3-regiopreference of this lipase (23). However, since *sn*-1,3 sites are available for the esterification reaction on all α/β-CX-MAG species, the preference for β-CX-MAG and α-C4-MAG species may reflect a lack of steric constraints conferred by the other α-CX-MAG species studied. The observations do not allow one to suggest that many MAG are preferred substrates over glycerol, because glycerol in our studies was immobilized, and the free glycerol in reactions systems is expected to be consider-

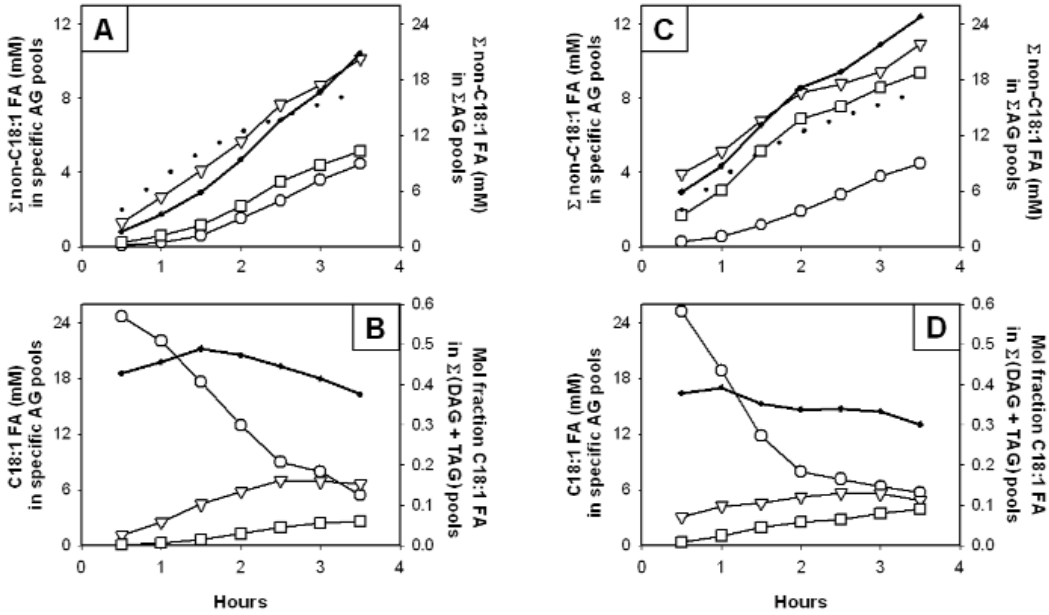

FIG. 7. Progress of esterification reactions between FA and C18:1-MAG. Figure legend is the same as for Figure 1 except the incorporation of non-C18:1 FA into AG appears in panel A for α-C18:1-MAG and Panel C for β-C18:1-MAG as substrates, and the recovery of esterified C18:1 from original C18:1-MAG is shown in panel B with α-C18:1-MAG, and panel D with β-C18:1-MAG as substrates.

ably less than the 50 mM CX-MAG employed. Lipase PS-30 selectivity toward various CX-MAG species was not consonant with enzyme selectivity toward CX-FA in esterification reactions, since α/β-C4-MAG was the most preferred MAG substrate, yet C4 FA was among the least reactive FA substrates. Furthermore, although C10 and C16 FA were preferred substrates for esterification, reactions rates were slowest when α-C10- and α-C16-MAG species were used as the alcohol cosubstrates. Our results on FA selectivity in esterification reactions with glycerol were similar to those reported earlier for reaction with octanol (24) with hexane as solvent.

Another facet of reaction selectivity regarding the original α/β-CX-MAG substrates is related to the extent that the original acyl group is channeled into the higher glycerides, DAG and TAG. Based on mol fraction CX (originating in α/β-CX-MAG) remaining in the Σ(DAG + TAG) pool by the end of the reaction period, reaction product selectivity favored channeling CX of the CX-MAG substrates into higher AG in the following (descending) order: α-C10-MAG ~ α-C16-MAG > α-C18:1-MAG > β-C10-MAG ~ β-C16-MAG ~ β-C18:1-MAG > α/β-C4-MAG. This pattern of product selectivity was not related to FA selectivity, as C18:1 was about twice as enriched in the higher AG pools as C4, although both of these FA are discriminated against by PS-30 lipase in esterification reactions. In addition, although C10 and C16 are preferred FA substrates for the PS-30 lipase, the α-CX-MAG derivatives of these FA were more conducive to enriching the higher AG in these same FA than were the corresponding β-CX-MAG species. Thus, acyl group selectivity of PS-30 lipase for incorporation into higher glycerides was dependent on whether the acyl group existed as FA or a FA-ester.

Although the recovered levels of total AG (Σ MAG + DAG + TAG) fell within a defined range of 21–28 mM for all reaction systems (Table 1), there were differences in which AG species became dominant by the end of the reaction period. Generally, higher glycerides were dominant in faster reaction systems, as expected. DAG were always a dominant AG pool accumulated, but TAG species also constituted a major pool for reaction systems employing all β-CX-MAG and α-C4-MAG substrates. In contrast, for α-C10- and α-C18:1-MAG substrates, TAG accumulated to modest levels, and only trace accumulation of TAG was observed for reactions with α-C16-MAG.

One of the primary motivations behind this study was to determine whether the nature (chain length and *sn*-glycerol site) of any acyl group residing along the glycerol backbone of MAG substrates would cause a shift in FA selectivity relative to that observed with glycerol. If so, this phenomenon could have a profound impact on the design of strategies to prepare AG of a specific and desired stereochemical configuration. Although we showed that the PS-30 lipase reacted at different rates (reflecting selectivity) with different α/β-CX-MAG species, the use of different MAG species generally did not affect the pattern of differential reactivity of the lipase with the FA substrates. Thus, there was a great deal of fidelity among the ordinal rankings of relative selectivity toward FA substrates in esterification steps giving rise to all AG species accumulating among the MAG and glycerol cosubstrates employed. The only exceptions, which constituted 3 of the 24 specific cases analyzed, were the selective enrichment of C14 and C18 FA in the MAG pool with α-C16-MAG as substrate, and a general suppression of >C8 FA incorporation into the

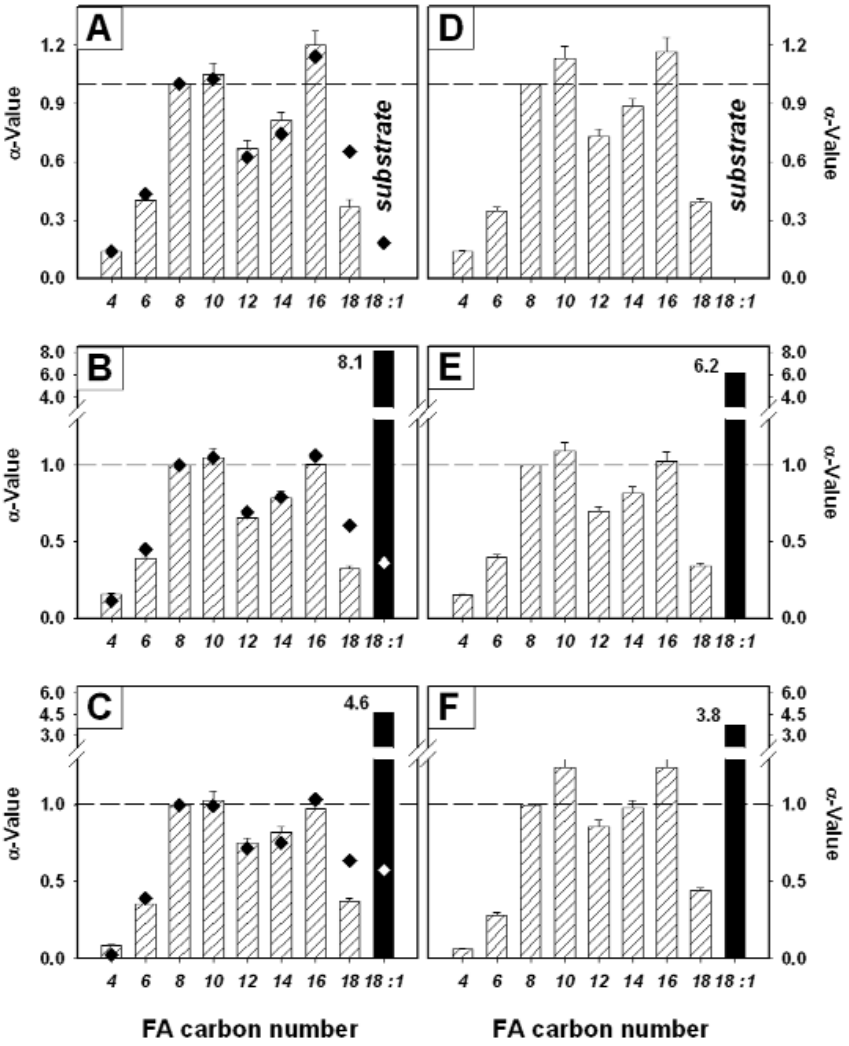

FIG. 8. Relative selectivity constants (α-values) with C18:1-MAG substrates for incorporation of FA into specific AG pools. Figure legend is the same as for Figure 2 except that reactions with α-C18:1-MAG are shown for incorporation of FA into MAG (panel A), DAG (panel B), and TAG (panel C) pools, and reactions with β-C18:1-MAG are shown for incorporation of FA into MAG (panel D), DAG (panel E), and TAG (panel F) pools. For abbreviation see Figure 1.

TAG pool with reactions employing α-C10- and α-C16-MAG species. The latter exception may be conferred largely by steric effects rather than intrinsic FA selectivity of the PS-30 lipase, since the preceding DAG species is likely to be of an α,α′-di-CX-DAG configuration, and final esterification with a large FA reactant may be difficult at the *sn*-2-glycerol site.

Thus, a generally applicable answer to the question "Does the acyl group and location in a MAG substrate influence FA selectivity for subsequent esterification steps?" is "No." Although this may effectively preclude a dimension of reaction control that may otherwise have been possible, the benefit of this condition is the simplicity in expecting lipase selectivity toward FA to be conserved throughout the incremental steps of assembling structured TAG. Whether this relationship holds for other lipases remains to be determined, and the companion paper (25) is a first step toward addressing this issue.

One last conclusion one can make from these studies is that MAG were preferred over FA as acyl donors for esterification/acyl-transfer reactions. This was gleaned from the 13–35 mM esterified glycerol recovered in the total AG pool at the initial sampling interval relative to the initial 50 mM CX-MAG present, whereupon only 0.4–13.9 mM non-CX FA was newly esterified in the total AG pool (Table 1). The preferential reactivity of acyl groups from MAG over FA may be conferred by the surfactant properties of MAG, the attendant partitioning into the microaqueous phase, and enhanced proximity to the lipase. The 15–37 mM CX released from the original MAG may become deposited in the FA pool or remain as an acyl-enzyme intermediate to participate in a reaction with another CX-MAG to yield a di-CX-MAG species. Indeed, this must have occurred to some extent, as the mol fraction of CX in the Σ(DAG + TAG) pool was >0.5 on two occasions,

with the α-C10- and α-C16-MAG species, and was slightly less than 0.5 when α-C18:1-MAG was the alcohol reactant.

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