Reaction Selectivity of *Rhizomucor miehei* Lipase as Influenced by Monoacylation of *sn*-Glycerol

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ABSTRACT: Reaction selectivities were determined in multicompetitive reactions mediated by *Rhizomucor miehei* (RM) lipase at 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 patterns of broad FA selectivity toward C8–C18 FA were generally observed for esterification into specific acylglycerol (AG) pools with the different α/β-CX-MAG cosubstrates. Exceptions were enrichment of C18 in the MAG pool with α-C16-MAG substrate, and a general suppression of C4/C6 FA reactivity and a specific discrimination toward >C8 FA incorporation into the TAG pool, both for reactions with α-C10- and α-C16-MAG. RM lipase selectivity toward MAG was in descending order: β-C18:1-MAG > α/β-C4-MAG ~ β-C10-MAG ~ β-C16-MAG > α-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 > β-C10-MAG ~ β-C16-MAG > α-C18:1-MAG > β-C18:1-MAG ~ α/β-C4-MAG. Aside from their characteristic FA selectivity, *Burkholderia cepacia* (PS-30) and RM lipases behaved similarly in terms of MAG selectivity as well as a general conservation of FA selectivity throughout the sequential steps of TAG assembly from FA and glycerol for processes designed to yield specifically structured TAG.

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KEY WORDS: Esterification, fatty acid, lipase, monoacylglycerol, *Rhizomucor miehei,* selectivity.

The prospect of using lipases to prepare uniquely functional "structured glycerides" for food and allied industries represents an opportunity to add value to basal lipid resources (1). The scientific literature is rife with examples of empirical or archival accounts of how lipids may be transformed by lipases into derivatives of enhanced functionality for specific applications. However, to fully exploit the synthetic power of lipases for these purposes, a comprehensive understanding of substrate and product selectivity patterns is required. This would enable the development of cogent strategies to use specific lipases to convert various lipid mixtures into desired structured glyceride derivatives.

One can envision the *de novo* assembly of structured TAG as a three-step, incremental process of selective acylation of each of the three *sn*-glycerol hydroxyl groups. Selectivity of enzymic reactions is quantitatively indexed in terms of specificity constants (k_{cat}/K_M, which is directly proportional to V_{max}/K_M), and such constants are applicable at any substrate concentration (2). Therefore, quantifying enzyme selectivity in terms of kinetic constants allows one to predict enzyme behavior under conditions where different substrate profiles and concentrations may be considered or anticipated.

Selectivity of several lipases toward a host of FA substrates has been quantified in terms of relative selectivity constants (often expressed in ratio format as relative α-values) by several research groups (3–11). In all cases, free alcohols or acetate esters of alcohol were employed as cosubstrates. What is not known is how progressive acylation of the *sn*-glycerol backbone, yielding different alcohol cosubstrates, may modulate lipase selectivity toward FA for subsequent esterification steps leading to structured TAG assembly. Alcohols are well known to affect lipase selectivity in many reactions using FA as cosubstrates (6,7,10–15).

Our companion paper (16) reported on the influence that various α/β-MAG species of defined acyl chain composition have on FA selectivity for progressive FA esterification reactions mediated by *Burkholderia cepacia* (Amano PS-30) lipase. Although the patterns of FA selectivity were generally conserved in reactions with different α/β-MAG species, there were some marked alterations of FA, MAG, and product selectivity in accumulating acylglycerols (AG). To provide contrast, a similar study to assess the influence of α/β-MAG species on FA selectivity of esterification reactions mediated by *Rhizomucor miehei* lipase was conducted.

MATERIALS AND METHODS

Materials. The lipase (RM lipase) from *R. miehei* (Chirazyme L-9, anion-exchange resin-fixed dry preparation, equivalent to "Lipozyme IM") was obtained commercially (Roche Diagnostics, Indianapolis, IN); the pH of the suspended RM lipase was 3.8. Sources of *sn*-glycerol-1(3)-monobutyrin (α-C4-MAG), *sn*-glycerol-1(3)-monocaprin (α-C10-MAG), *sn*-glycerol-1(3)-monopalmitin (α-C16-MAG), *sn*-glycerol-1(3)-monoolein (α-C18:1 MAG), *sn*-glycerol-2-monobutyrin (β-C4-MAG), *sn*-glycerol-2-monocaprin (β-C10-MAG), *sn*-glycerol-2-monopalmitin (β-C16-MAG), *sn*-glycerol-2-monoolein (β-C18:1-MAG), and other reagents were as identified in the companion paper (16).

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Competitive enzyme reactions. The reaction system routinely used was identical to that described in the companion paper (16), and consisted of 50 mM of a single CX-MAG species (where X is the *n*-acyl chain length) 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 640 mM. Glycerol, when used, was immobilized on silica gel prior to introduction into the reaction mixture. Reactions were carried out in *n*-hexane (20 mL), and water activity (a_w) was poised at 0.19 by the salt hydrate pair (1.2 g each) of anhydrous Na_2HPO_4 and $Na_2HPO_4 \cdot (H_2O)_2$ (16).

Sampling and analysis. Reaction mixtures were preincubated at 35°C for 30 min, and RM lipase (100 mg) was added to initiate the reaction. Subsamples of 0.35 mL were withdrawn over predetermined intervals, centrifuged to remove particulate matter, resolved into MAG, DAG, and TAG components, and subjected to FAME analysis as described in the companion paper (16).

FA selectivity and kinetic analysis. Competitive factors (relative selectivity constants or α-values) among multiple FA substrates were determined based on the application of conventional enzyme kinetics (2,3) and described more fully in the companion paper (16). Briefly, α-values were determined as slopes from log–log plots characterizing reaction progress curves, relative to the reactivity of a reference substrate (C8 FA in this study). The α-value of the reference substrate (C8 FA) was taken as 1.0, and competing FA substrates with a greater α-value by comparison were more reactive.

Average initial reaction rates (v_o') were calculated on the basis of the first interval of analysis of FA esterified into the AG pool (MAG + DAG + TAG), because the initial periods of reaction could not be linearized in many cases. Although these values do not represent true initial velocities (v_o), calculated v_o' serve the purpose of allowing a comparison of lipase selectivity among different MAG species. Analyses of FA selectivity and overall esterification reaction rates were also done for reaction mixtures employing the full set of FA (C4–C18, C18:1; 720 mM FA total) with 50 mM glycerol (instead of MAG) for comparison. In some cases, kinetic analyses 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.

RESULTS AND DISCUSSION

Reactions with C4-MAG species. Esterification reactions by RM lipase were about twice as fast (based on the initial time point analyzed) with either α- or 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. Surprisingly, non-C4 FA substrates accumulated in the MAG pool as quickly as in the TAG pool (Figs. 1A, C), whereas for reactions mediated by PS-30 lipase, there was an

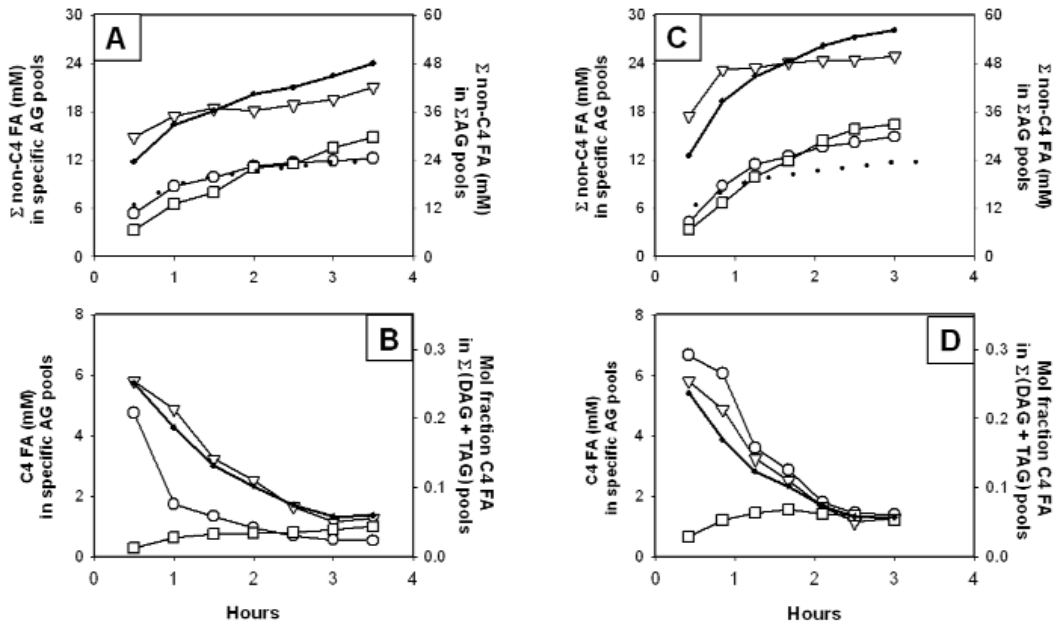

FIG. 1. Progress of esterification reactions between FA and C4-MAG. Incorporation of non-C4 FA into specific acylglycerol (AG) pools (open symbol plots, left ordinate) and total non-C4 FA esterified in all AG pools (bold line plots, right ordinate; reaction with glycerol shown as dotted line) is shown 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, estimate)[a]		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[b]	Non-CX FA	CX	ΣAG[b]
Glycerol		26	1.0	12.9[c]	—	—	23.6[c]	—	—
C4-MAG	(α)	47	1.8	23.5	10.9	21.6 *0.46, 0.48, 0.06*	48.0	2.82	29.1 *0.44, 0.38, 0.18*
	(β)	50	1.9	25.1	13.1	23.9 *0.46, 0.49, 0.06*	56.2	3.87	35.2 *0.46, 0.37, 0.17*
C10-MAG	(α)	3.5	0.14	1.76	33.0	32.1 *0.91, 0.08, 0.00*	13.2	24.2	25.6 *0.52, 0.46, 0.01*
	(β)	46	1.8	23.2	16.1	24.8 *0.48, 0.46, 0.06*	36.7	6.70	24.4 *0.41, 0.40, 0.19*
C16-MAG	(α)	3.9	0.15	1.93	7.74	7.92 *0.79, 0.20, 0.01*	5.59	19.2	18.8 *0.69, 0.31, 0.01*
	(β)	51	1.9	25.3	19.8	25.6 *0.39, 0.46, 0.15*	39.9	8.49	25.6 *0.36, 0.39, 0.25*
C18:1-MAG	(α)	40	1.6	20.1	7.40	18.6 *0.54, 0.45, 0.01*	34.1	2.61	21.7 *0.44, 0.41, 0.14*
	(β)	62	2.4	31.2	7.44	24.0 *0.46, 0.46, 0.07*	46.9	2.26	28.0 *0.40, 0.43, 0.16*

[a]Reaction velocity was calculated as an average velocity for the entire reaction period and does not represent a true v_o.
[b]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.
[c]Reaction mixtures with glycerol employed all FA within the series of C4–C18, C18:1. There are no "non-CX" or "CX" FA species in this case.

obvious lag period before non-C4 FA accumulated in MAG (16). Analogous observations were made for all other reaction systems evaluated with RM lipase, indicating a general difference in catalytic patterns between PS-30 and RM lipases.

As reactions were allowed to progress, C4 became diminished in both the MAG and DAG pools while becoming enriched in the TAG pool (Figs. 1B, D). The facile accumulation of non-C4 in MAG and the presence of C4-containing DAG as a dominant AG pool early in the reaction indicate that non-C4-MAG may be as likely to accumulate through C4-group removal from a mixed-acid C4-containing DAG species, as by C4-group removal from α/β-C4-MAG followed by esterification of the resulting glycerol with a non-C4 FA. The latter alternative was suggested to be more likely in reactions mediated by PS-30 lipase with all α/β-MAG species (16). Key differences in behavior were that for RM lipase reactions with α/β-C4-MAG, DAG levels were 10–12 mM, and free glycerol was 26–28 mM at the first sampling interval [compared with 3–6 mM DAG and 34–37 mM free glycerol for PS-30 lipase reactions (16)] for similar levels (range of 11–16 mM) of C4 remaining esterified in the ΣAG pools (Table 1, Figs. 1A–D; also Table 1 in Ref. 16). Furthermore, RM lipase is known to react with different AG species faster than glycerol in (inter)esterification processes (17), and RM lipase selectively hydrolyzes tributyroylglycerol at acid pH (18). These facts lend further support to the postulated origin of non-C4-MAG species being C4-containing DAG.

The mol fraction of C4 accumulating in the combined DAG and TAG pools declined from 0.2–0.3 to <0.1 throughout the reaction period, and accounted for only 5–8% of the original C4 existing as α/β-C4-MAG (Figs. 1B, D). These observations are evidence of exclusion of C4 from these two AG pools through product selectivity of the reaction, perhaps manifested as the equilibrium position of butyroylglycerol species favoring net hydrolysis relative to the other FA-glycerol species existing in the reaction systems.

RM lipase selectivity for incorporation of non-C4 FA into the discrete AG pools for reactions with α/β-C4-MAG exhibited broad selectivity for FA C8–C18 (Fig. 2). The ordinal patterns of FA selectivity were similar to esterification reactions with glycerol as alcohol cosubstrate (diamond insets on Fig. 2A–C). The incorporation of C4 into TAG yielded comparatively large α-values, appearing as black bars in Figure 2 (panel F). However, these α-values were likely conferred only through the channeling of C4-MAG into the TAG pool through progressive esterification, and not by direct esterification reactions using C4 FA. In fact, these α-values were the smallest observed by us for CX originating from α/β-CX-MAG and entering the TAG pool and are consistent with the selective exclusion of C4 from the accumulated AG species mediated by RM lipase.

Reactions with C10-MAG species. Relative to reaction rates with glycerol as alcohol cosubstrate, esterification reactions by RM lipase were only 15% as fast with α-C10-MAG (Fig. 3A) and almost twice as fast (based on the initial time point) with β-C10-MAG (Fig. 3C) as alcohol cosubstrate (Table 1). The ready incorporation of non-C10 FA into all three AG pools (MAG, DAG, and TAG) occurred with β-C10-MAG by the first interval of analysis, whereas reactions with α-C10-MAG exhibited only notable non-C10 FA incor-

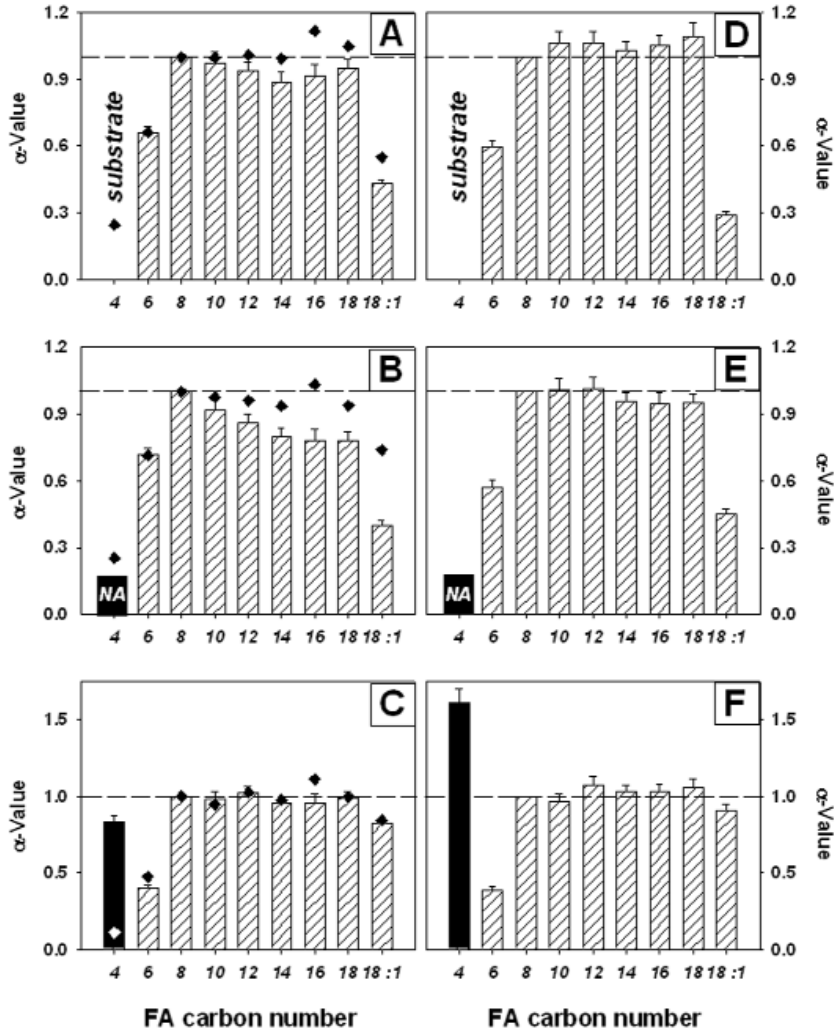

FIG. 2. Relative selectivity constants (α-values) with C4-MAG substrates for incorporation of FA into specific AG pools. Reactions with α-C4-MAG are shown for incorporation of FA into MAG (panel A), DAG (panel B), and TAG (panel C) pools. Reactions with β-C4-MAG are shown for incorporation of FA into 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. "NA" indicates that data for C4 from the original C4-MAG substrate did not fit the kinetic model to allow estimation. For abbreviation see Figure 1.

poration into the DAG pool at this point in the reaction. Furthermore, a stark contrast between these two reaction systems was that almost no TAG accumulation occurred with α-C10-MAG as cosubstrate (Figs. 3A, B), whereas TAG accumulated readily with β-C10-MAG (Figs. 3C, D) as cosubstrate.

As reactions were allowed to progress, C10 became diminished in the MAG and enriched in the DAG pool with α-C10-MAG (Fig. 3B). Recovery of C10 esterified in the ΣAG pools at the initial sampling interval was 33 mM of the initial 50 mM (Table 1), indicating limited net hydrolysis of C10 or exchange with the FA pool. In contrast, C10 became diminished in both the MAG and DAG pool, while becoming enriched in the TAG pool throughout the entire reaction period with β-C10-MAG as substrate (Fig. 3D). The mol fraction of C10 in combined Σ(DAG + TAG) pools was maintained at 0.6–0.7 and continually declined from 0.4 to <0.2 for the reaction period for reaction systems based on α-C10-MAG and β-C10-MAG cosubstrates, respectively (Figs. 3B, D). This pattern of enrichment of C10 in DAG in reactions with α-C10-MAG is consistent with the conservation of the original α-C10-MAG configuration coupled to the addition of other C10 FA residues leading to DAG accumulation.

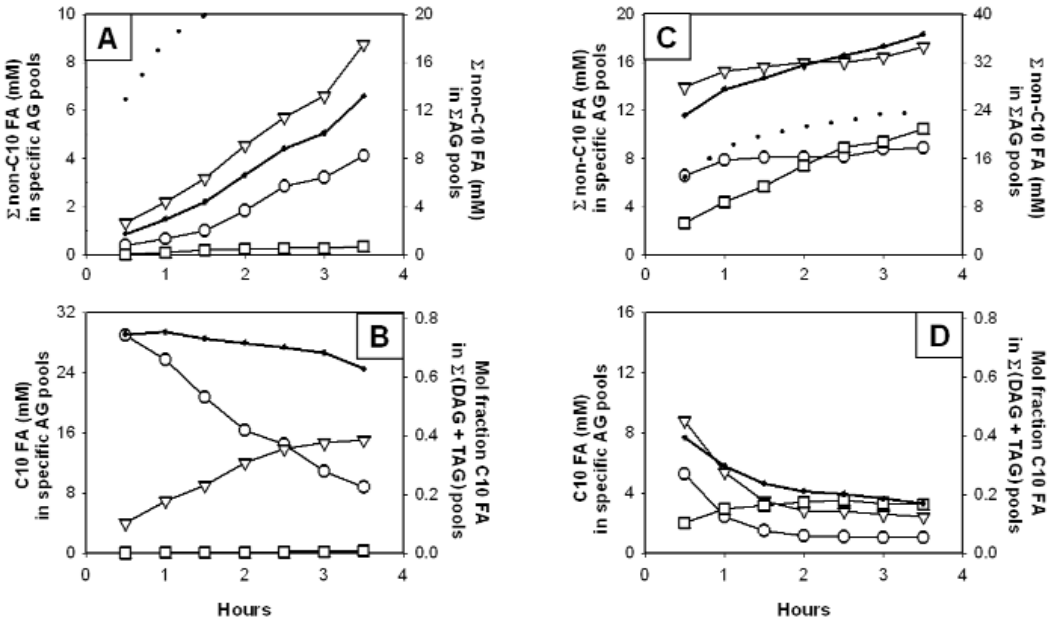

FIG. 3. Progress of esterification reactions between FA and C10-MAG. Figure legend is the same as for Figure 1 except the incorporation of non-C10 FA into AG appears in panel A for α-C10-MAG and panel C for β-C10-MAG as substrates, and the recovery of esterified C10 from original C10-MAG is shown in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

RM lipase selectivity for non-C10 FA incorporation into the discrete AG pools for reactions with α/β-C10-MAG exhibited a broad selectivity for FA C8–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. However, reactivity appeared to be suppressed toward FA C4–C6 with α-C10-MAG relative to glycerol or β-C10-MAG as cosubstrate. More importantly, it appeared that steric constraints were imposed on reaction systems with α-C10-MAG for steps leading to the accumulation of DAG and especially TAG, where selectivity for C12–C18 FA was diminished by 50–75% (Figs. 4A–C). In contrast, there was no perceivable change in FA selectivity as reactions progressed through successive esterification steps with β-C10-MAG (Figs. 4D–F) or glycerol (Figs. 4A–C) as cosubstrate.

The incorporation of C10 into higher glycerides (DAG and TAG) could be kinetically characterized by α-values in two cases (Figs. 4B, F). The large α-values for C10 entering the DAG pool with α-C10-MAG as cosubstrate (Fig. 4B) coupled with the 66% retention of esterified C10 (Table 1) and high mol fraction of C10 in the Σ(DAG + TAG) pool (Fig. 3B) imply that accumulated DAG species arise in part from the acyl transfer of C10 from one C10-MAG species to another in this reaction system. The α-values observed for C10 incorporation into the TAG pool (Fig. 4F) are likely conferred by simple channeling of β-C10-MAG into the TAG pool *via* esterification to non-C10 FA, since the mol fraction of C10 in the Σ(DAG + TAG) pool approached ≤0.2 (Fig. 3D).

Reactions with C16-MAG species. Similar to reactions with corresponding C10-MAG species (Fig. 3), esterification reactions by RM lipase were lethargic with α-C16-MAG (Fig. 5A) and occurred readily with β-C16-MAG (Fig. 5C) as cosubstrate (Table 1), with non-C16 FA being readily incorporated into all AG species in the latter case. α-C16-MAG reaction systems behaved anomalously since only 8 mM (of 50 mM) originally esterified C16 and glycerol was recovered in ΣAG pools at initial sampling, and the trend in C16-MAG levels was unusual (Fig. 5B).

For the β-C16-MAG-based reaction mixture, the rapid appearance of non-C16 FA in all TAG and MAG, and C16-containing DAG at initial sampling (Figs. 5C, D) imply that non-C16-MAG could be formed from both esterification of free glycerol (calculated at 24 mM) and hydrolysis of C16-containing DAG (total DAG at 11–12 mM).

Mol fraction analysis indicated a selective enrichment of C16 in the Σ(DAG + TAG) pools (0.6–0.7) for α-C16-MAG reaction systems and an exclusion of C16 from higher AG (declining to 0.2) for β-C16-MAG reaction systems (Figs. 5B, D). This is consistent with the selective relative enrichment of C16 in DAG through reaction of α-C16-MAG as both acyl donor and alcohol cosubstrate.

RM lipase selectivity for incorporation of non-C16 FA into AG pools arising from α/β-C16-MAG exhibited a broad preference for FA C8–C16, with exceptions noted for the α-C16-MAG reaction system (Fig. 6). Enhanced selectivity for C18 FA incorporation into the MAG pool was observed (Fig. 6A), as was also noted for reactions mediated by PS-30 lipase (16). A likely explanation is the hydrolysis of the corresponding C16,CX-DAG species, despite rather low levels of DAG accumulated in this reaction system (Table 1; Figs. 5A, C). Other exceptions were the diminished reactivity of C4–C6 FA

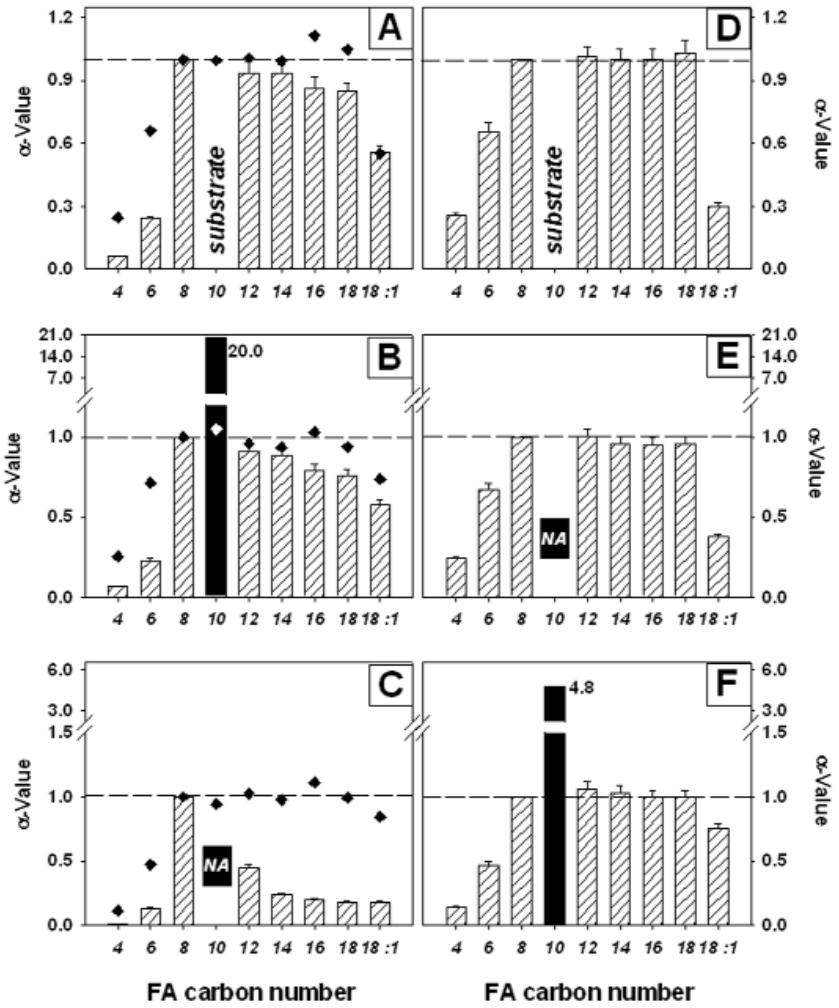

FIG. 4. Relative selectivity constants (α-values) with C10-MAG substrates for incorporation of FA into specific AG pools. Figure legend is the same as for Figure 2 except that reactions with α-C10-MAG are shown for incorporation of FA into MAG (panel A), DAG (panel B), and TAG (panel C) pools, and reactions with β-C10-MAG are shown for incorporation of FA into MAG (panel D), DAG (panel E), and TAG (panel F) pools. For abbreviations see Figure 1.

and C10–C18/C18:1 FA for incorporation into the DAG and especially TAG pool (Figs. 6B, C), trends also noted for reactions with α-C10-MAG (Figs. 4B, C).

The large α-value observed for C16 incorporation into the DAG for α-C16-MAG-based reactions (Fig. 6B) can be explained by the enhanced reaction selectivity for C16 incorporation into higher glycerides mentioned earlier. The relatively large α-value for deposition of C16 into the TAG pool of β-C16-MAG-based systems (Fig. 6F) is likely conferred by simple channeling of β-C16-MAG into the TAG pool through progressive esterification reactions with non-C16 FA.

Reactions with C18:1-MAG species. Esterification reactions by RM lipase were about 50% faster with α-C18:1-MAG (Fig. 7A) and 150% faster with β-C18:1-MAG (Fig. 7C) relative to reactions with free glycerol, based on non-C18:1 FA incorporation into all AG pools (Table 1). Qualitatively, reaction patterns were quite similar between the two α/β-C18:1-MAG substrate systems in that at the initial time interval, non-C18:1-MAG species were abundant and DAG constituted the dominant AG pool, for C18:1 and non-C18:1 FA populations (Fig. 7). The subtle differences between these two reaction systems were that both C18:1 and non-C18:1 FA accumulated in TAG at a slower rate for α-C18:1-MAG (Figs. 7A, B) than for β-C18:1-MAG (Figs. 7C, D) reaction systems. In both cases, the exclusion of C18:1 from the accumulating Σ(DAG + TAG) pool was evident as a progressive decline in the mol fraction of C18:1 FA from 0.2–0.3 to <0.1 (Figs. 7B, D). At the initial time point, DAG were present at 8–11 mM, and free glycerol was calculated to be 26–31 mM, indicating that non-C18:1-MAG species were likely derived from both hydrolysis of C18:1-MAG followed by esterification as well as hydrolysis of C18:1-containing DAG.

RM lipase selectivity for incorporation of non-C18:1 FA into the discrete AG pools for reactions with α/β-C18:1-

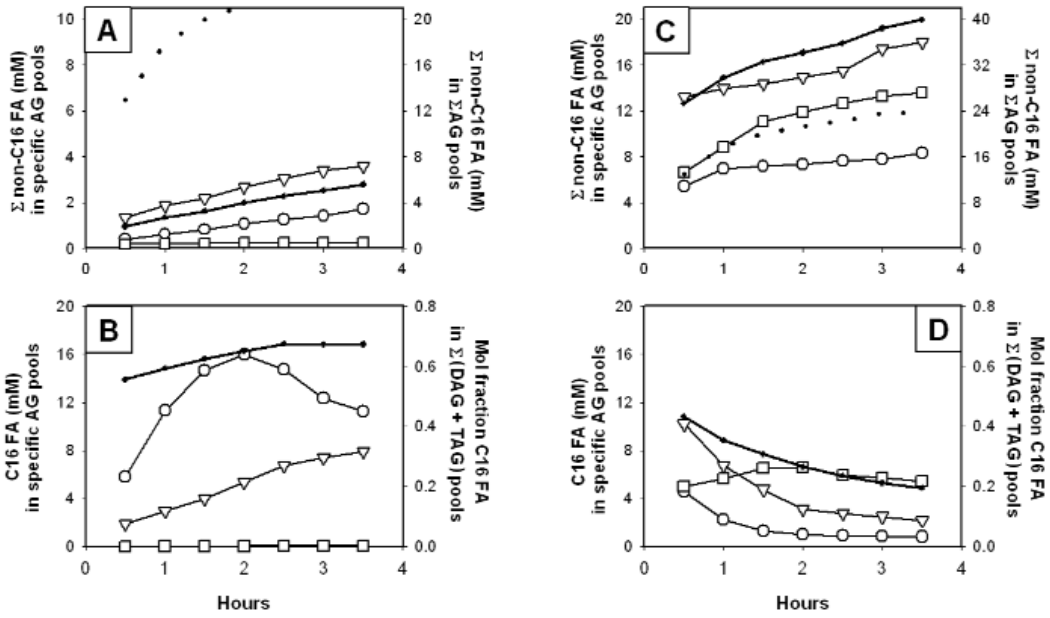

FIG. 5. Progress of esterification reactions between FA and C16-MAG. Figure legend is the same as for Figure 1 except the incorporation of non-C16 FA into AG appears in panel A for α-C16-MAG and panel C for β-C16-MAG as substrates, and the recovery of esterified C16 from original C16-MAG is shown in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

MAG exhibited a similar and broad preference for FA C8–C16 (Fig. 8). The patterns of FA selectivity in esterification reactions with α/β-C18:1-MAG were similar to those with glycerol except that reactions with the MAG substrates were discriminatory toward and about half as reactive with C18 FA than with glycerol (diamond insets on Figs. 8A–C). The α-values for incorporation of C18:1 into TAG (Figs. 8C, F) are likely conferred primarily through the channeling of C18:1-MAG into these AG pools by progressive esterification with non-C18:1 FA, since mol fraction analysis indicated that C18:1 FA was excluded from this pool (Figs. 7B, D).

General discussion. The overall objective of this work was to establish the patterns of reaction selectivity of RM lipase in terms of successive steps in assembling TAG from MAG or glycerol and FA to provide a comparison and contrast with the patterns established for PS-30 lipase (16). The same caveats offered in the preceding paper also apply to the present study. The observed selectivity in the reaction systems studied is a reflection of intrinsic lipase selectivity as modulated by the specific choices of solvent, salt hydrate, and a_w that were made, as well as by physical and interfacial properties conferred by reaction components and how these properties may change during reaction progress. The most important aspect of this work to consider is that determinations of reaction selectivity are founded on net flow of FA (esterification) into accumulating AG pools originating from a specific MAG species, despite the coexistent and sometime competing processes of acyl migration, interesterification, and hydrolysis. As stated in the related study (16), the limit of our studies to a time frame of 3.5 h minimizes any role that acyl migration may have on our results as acyl migration rates for β-MAG and α,β-DAG are on the order of a <5%/h in hexane (19,20).

On the basis of initial reaction rates (Table 1), RM lipase selectivity toward MAG species was in the following descending order: β-C18:1-MAG > α/β-C4-MAG ~ β-C10-MAG ~ β-C16-MAG > α-C18:1-MAG > *(glycerol)* > α-C10-MAG ~ α-C16-MAG. This pattern is consistent with the known *sn*-1,3 regiopreference of this lipase (21), as it was for PS-30 lipase (16,21), since the β-CX-MAG species would be considered easier to react with the enzyme to yield TAG. The ready depletion of CX- from β-CX-MAG presumably through hydrolysis and especially for C4 and C18:1 species (Table 1) is not easily reconciled on the basis of the *sn*-1,3 regiopreference of RM lipase (21). However, hydrolytic regioselectivity is conventionally studied on TAG species, and it is possible that reactivity toward lower glycerides is less constrained. In fact, numerous studies have shown RM lipase to be useful in transforming secondary alcohols and their esters (22 and references therein). TAG accumulated readily in reactions employing β-CX-MAG, but only for reactions with α-C4-MAG and α-C18:1-MAG among the α-CX-MAG series. The lack of TAG accumulation in cases of α-C10-MAG and α-C16-MAG represents a lack of reactivity, which is a direct reflection of enzyme selectivity and which may be founded on steric constraints as advanced previously (16).

RM lipase selectivity toward various CX-MAG species was not consonant with enzyme selectivity toward CX-FA in esterification reactions, since α/β-C4-MAG and α/β-C18:1-MAG were among the most preferred MAG substrates, yet C4 and C18:1 FA were among the least reactive FA substrates. This was similar to the observations and conclusion made for PS-30

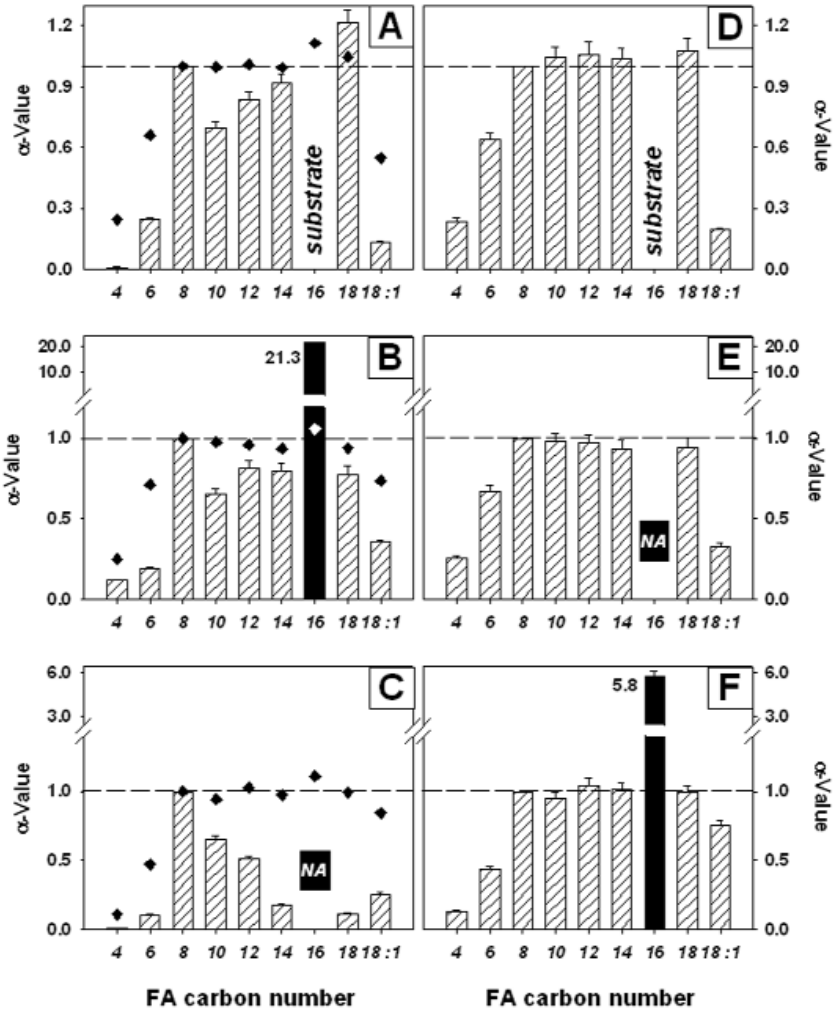

FIG. 6. Relative selectivity constants (α-values) with C16-MAG substrates for incorporation of FA into specific AG pools. Figure legend is the same as for Figure 2 except that reactions with α-C16-MAG are shown for incorporation of FA into MAG (panel A), DAG (panel B), and TAG (panel C) pools, and reactions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

lipase reactions (16). Acyl groups esterified as AG must have dual roles in lipase reaction selectivity; they may bind at the scissile FA binding site as acyl donor, and they may bind at the alcohol cosubstrate binding site, as there are discrete binding sites for both cosubstrates (23). RM lipase, like other lipases, exhibits a characteristic pattern of selectivity among a series of *n*-alkyl and substituted *n*-alkyl alcohols (10–13,15). The preference of RM lipase for α/β-C4-MAG and α/β-C18:1-MAG in esterification processes may reflect the ability of the lipase to easily bind/recognize these acyl groups, since it prefers tributyrin and olive oil as TAG substrates for hydrolysis (16). These same FA (C4 and C18:1) may be preferentially excluded from accumulating glycerides since they were not preferentially esterified (Figs. 2, 4, 6, 8), and they were most extensively lost from the original α/β-CX-MAG species used (Table 1).

Both RM and PS-30 lipases preferred the same MAG species, α/β-C4-MAG and α/β-C18:1-MAG, indicating that smaller and/or more flexible MAG substrates may be preferred by lipases as alcohol cosubstrates in general over those with long, saturated acyl chains. However, such a hypothesis would require further experimental validation.

Reactivity of RM lipase with systems employing FA with α-C16-MAG yielded an anomalous reaction pattern in terms of the transient fate of C16 esterified in the MAG pool. This was also observed for reactions with PS-30 lipase, and we suggest that physical effects, such as solubility/fluidity constraints, may be a major contributor to, and partially confound, our assessments of reaction selectivity with α-C16-MAG in both cases (16).

Product selectivity for RM lipase-mediated reactions was also indexed by the extent of channeling of the CX acyl groups derived from the original α/β-CX-MAG substrates into the higher glycerides, DAG and TAG. Based on mol fraction analysis, the relative degree of enrichment (product

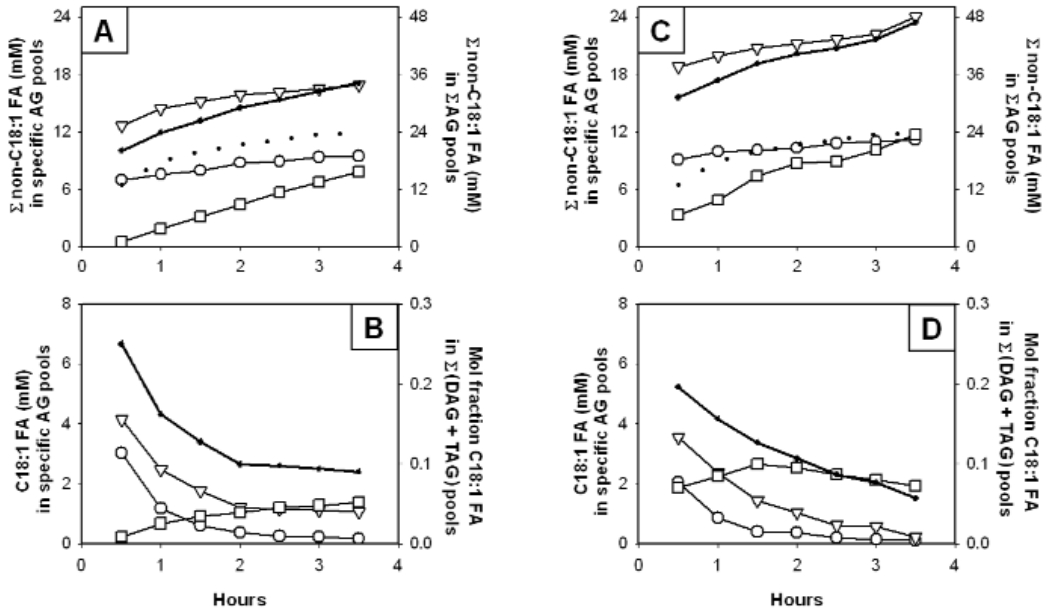

FIG. 7. Progress of esterification reactions between FA and C18:1-MAG. Figure legend is the same as for Figure 1 except the incorporation of non-C18:1 FA into AG appears in panel A for α-C18:1-MAG and panel C for β-C18:1-MAG as substrates, and the recovery of esterified C18:1 from original C18:1-MAG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

selectivity) of CX originating from CX-MAG into higher AG was in descending order: α-C10-MAG ~ α-C16-MAG > β-C10-MAG ~ β-C16-MAG > α-C18:1-MAG > β-C18:1-MAG ~ α/β-C4-MAG. With the exception of the position of α-C18:1-MAG, this ranking was similar to reactions mediated by PS-30 lipase (16). The order of α/β-CX-MAG on this list was inversely proportional to the extent of reaction taking place as measured by the mM level of non-CX FA esterified by the end of the reaction period (*cf.* Tables 1, this paper, and in Ref. 16). Furthermore, the degree of enrichments (mol fraction) of CX from α/β-CX-MAG in Σ(DAG + TAG) was generally less for reactions mediated by RM lipase than for those mediated by PS-30 lipase. This was associated with greater extents of reaction in the former case (34–56 mM non-CX FA incorporated) than in the latter case (21–44 mM non-CX FA incorporated), not including the slow-reacting α-C10-MAG and α-C16-MAG systems. Although an increasing extent of incorporation of non-CX FA will "dilute" CX in accumulating DAG and TAG pools, a modest degree of product selectivity was conferred by both enzyme reactions. The relative α-values for CX were C16 > C10 > C18:1 for enrichment in the TAG pool derived from the corresponding β-CX-MAG for reactions by both enzymes. This was observed for a similar range of non-CX FA incorporation into the AG pool (25–31 mM accumulation for PS-30 lipase, and 37–47 mM for RM lipase) and with similar extents of TAG accumulation (4–6 mM).

The recovered levels of total AG (Σ MAG + DAG + TAG) at the end of reaction periods fell within a range of 19–35 mM (Table 1). As may be expected, the levels of non-CX FA esterified in reactions with CX-MAG were directly related to the levels of AG and proportion of higher glycerides (DAG + TAG) accumulated by the end of the reaction period. Although reactions with PS-30 lipase were shown to be selective for MAG over FA as acyl donors for esterification/acyl-transfer reactions (16), reactions with RM lipase appeared to readily employ both (MAG and FA) acyl donor pools. This difference was manifested in the patterns and the suggested origins of non-CX-MAG accumulation from CX-MAG, which occurred earlier in reactions with RM lipase than with PS-30 lipase. In both cases, the facile reactivity of acyl groups from MAG (used at 50 mM) over FA (used at 640 mM total) may be conferred by the surfactant properties of MAG and its partitioning into the microaqueous phase, proximal to the lipase.

In general, RM lipase reactions exhibited broader FA selectivity than did PS-30 lipase reactions (16). In addition, the nature (chain length and *sn*-glycerol site) of the acyl group residing along the glycerol backbone of MAG substrates had only a modest influence on FA selectivity relative to that observed with glycerol in reactions mediated by RM lipase. The specific perturbations in RM lipase reaction selectivity were a general suppression of >C8 FA reactivity for incorporation into the DAG and especially TAG pool when α-C10-, C16-MAG substrates were used, also similar to the findings with PS-30 lipase. Again, steric constraints are implicated by these findings, and these constraints may reflect the *sn*-1,3 regioselectivity of these lipases. It would be interesting to contrast this behavior with that of a nonregioselective lipase to see whether the proposed steric constraints are diminished.

Thus, a high degree of fidelity of relative selectivity toward FA substrates in sequential esterification steps for both PS-30 and RM lipases implies that FA selectivity for the multiple steps of TAG assembly from glycerol and FA is generally conserved throughout the process. Consequently, we

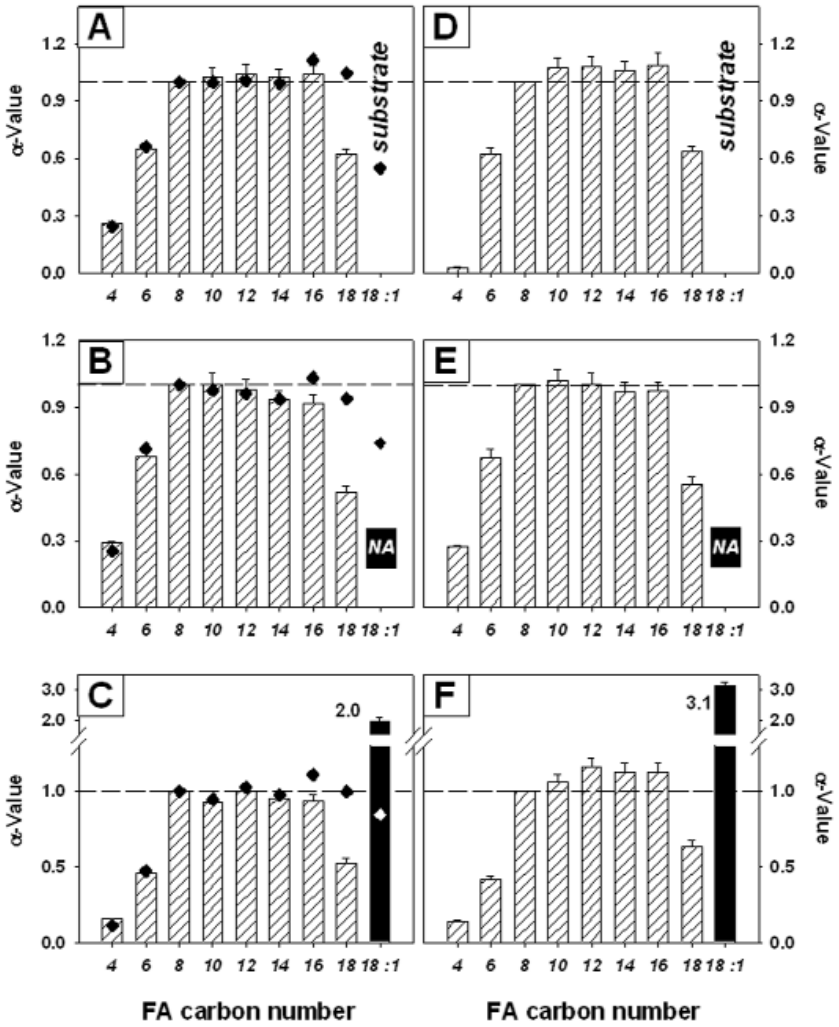

FIG. 8. Relative selectivity constants (α-values) with C18:1-MAG substrates for incorporation of FA into specific AG pools. Figure legend is the same as for Figure 2 except that reactions with α-C18:1-MAG are shown for incorporation of FA into MAG (panel A), DAG (panel B), and TAG (panel C) pools, and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

would predict the accumulating MAG, DAG, and TAG pools to have a similar FA profile in reactions employing glycerol and FA using PS-30 and RM lipases. This would appear to simplify the design process in assembling a structured TAG from basic building blocks in that it can be based on the characteristic FA selectivity of a lipase in reactions with free glycerol as alcohol cosubstrate, for which there are ample data reported in the literature (5–7,11,16). We have shown this relationship to apply to two specific and widely used lipases, and further validation among other common lipases remains to be seen.

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