Retinol esterification in bovine retinal pigment epithelium: reversibility of lecithin:retinol acyltransferase

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Esterification of all-*trans*-retinol is a key reaction of the vertebrate visual cycle, since it produces an insoluble, relatively non-toxic, form of the vitamin for storage and supplies substrate for the isomerization reaction. CoA-dependent and -independent pathways have been described for retinol esterification in retinal pigment epithelium (RPE). The CoA-independent reaction, catalysed by lecithin:retinol acyltransferase (LRAT) was examined in more detail in this study. Addition of retinol to RPE microsomes results in a burst of retinyl ester synthesis, followed by a rapid apparent cessation of the reaction. However, [^{3}H]retinol, added when retinyl ester synthesis has apparently ceased, is rapidly incorporated into retinyl ester without a net increase in the amount of ester. The specific radioactivities of [^{3}H]retinol and [^{3}H]retinyl ester reach the same value. [^{14}C]Palmitate from palmitoyl-CoA is incorporated into pre-existing retinyl ester in the absence of net ester synthesis, too. These exchange reactions suggest that the reaction has reached equilibrium at the plateau of the progress curve and that only the accumulation of retinyl ester, and not its synthesis, has stopped during this phase of the reaction. Studies with geometrical isomers of retinol revealed that the rate of exchange of all-*trans*-retinol with all-*trans*-retinyl esters was about 6 times more rapid than exchange of 11-*cis*-retinol with 11-*cis*-retinyl ester. This is the first demonstration of the reversibility of LRAT and the first example of stereospecificity of retinyl ester synthesis in the visual system. Reversal of the LRAT reaction could contribute to the mobilization of 11-*cis*-retinol from 11-*cis*-retinyl ester pools.

INTRODUCTION

Retinol esterification has been considered a detoxifying reaction, since it converts the vitamin into an insoluble ester that can be stored without the adverse manifestations of the more soluble retinol (for a recent review, see Blomhoff et al., 1990). In liver, the dynamic equilibrium between the intracellular retinyl ester pool and retinol-binding-protein (RBP)-bound plasma retinol is an important component of plasma retinol homoeostasis, indicating that retinyl esters play more than a passive role in vitamin A metabolism. Recent findings in the visual system indicate that retinyl esters play an additional active role in the visual cycle. In retinal pigment epithelium (RPE) the key reaction of the visual cycle, regenerating the 11-*cis* configuration of vitamin A, has been shown to result from conversion of all-*trans*-retinyl ester into 11-*cis*-retinol, a concerted reaction involving isomerization and ester hydrolysis (Deigner et al., 1989). Esterification of all-*trans*-retinol in the visual system thus plays an active role in the regeneration cycle, since it supplies substrate for the isomerization reaction [see Saari (1990) and Rando (1991) for recent reviews of the visual cycle].

Retinyl ester in RPE and other tissues is synthesized in CoA-dependent and -independent reactions, the former catalysed by acyl-CoA:retinol acyltransferase (ARAT) and the latter by lecithin:retinol acyltransferase (LRAT) (MacDonald and Ong, 1988; Saari and Bredberg, 1988, 1989; Yost et al., 1988). In our studies of retinyl ester synthesis in bovine RPE we have noted that the CoA-independent reaction is characterized by an extremely rapid apparent cessation of the reaction (for an example, see Figure 1 of Saari and Bredberg, 1988). We analysed the events leading to the generation of this plateau in the reaction progress curve in more detail. Exchange studies demonstrate that retinyl ester synthesis continues during the plateau phase without an increase in the amount of retinyl ester. The results suggest that the LRAT reaction has reached equilibrium, a feature that may have physiological significance for the mobilization of 11-*cis*-retinol from retinyl ester pools.

EXPERIMENTAL

Materials

Sources and purifications of all-*trans*-retinol and 11-*cis*-retinol have been given in previous papers (Saari and Bredberg, 1988, 1989). The term 'retinol' in the present paper refers to all-*trans*-retinol unless otherwise designated. [^{3}H]Retinol, [^{14}C]palmitoyl-CoA and [^{14}C]palmitate were purchased from DuPont–New England Nuclear. [^{3}H]11-*cis*-retinol was produced by reduction of 11-*cis*-retinaldehyde with NaB^3H_4. 11-*cis*-Retinaldehyde was provided through the generosity of the National Eye Institute. Palmitoyl-CoA, PC, lysoPC and phenylmethanesulphonyl fluoride were purchased from Sigma Chemical Corp. (St. Louis, MO, U.S.A.), and the lipids were purified by t.l.c. or h.p.l.c., if necessary (Saari and Bredberg, 1989).

Assay for retinol esterification

Conditions for LRAT assays have been given by Saari and Bredberg (1989, 1990). Departures from these conditions, especially in retinol concentrations, are noted in the text or Figure legends. Assays were conducted for various times as indicated in the legends to the Figures. Initial rates were determined from 0.5-, 1- or 2-min assays. Longer assays were used for other

Abbreviations used: ARAT, acyl-CoA:retinol acyltransferase; LRAT, lecithin:retinol acyltransferase; PC, phosphatidylcholine; RBP, retinol-binding protein; RPE, retinal pigment epithelium.

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experiments. Dithiothreitol was included in all assays at 1 mM unless otherwise designated. BSA was present in all reaction mixtures at a concentration of 60 μM, except for the experiments involving quantification of non-esterified fatty acids where ovalbumin was substituted.

Preparation of microsomes from bovine RPE

Preparation and storage of microsomes have been described by Saari and Bredberg (1990). Microsomes prepared from calf and adult bovine eyes were used in this study.

Assay for fatty acids

Non-esterified fatty acids, retinol and retinyl esters were extracted from esterification reaction mixtures. To a 200 μl reaction volume was added 1 ml of methanol, 3 ml of light petroleum (b.p. 35–60 °C) and 1 ml of 50 mM K_2CO_3, pH 10. After mixing, the upper phase was removed and extracted with 1 ml of water, which was added to the original lower phase. Retinol and retinyl ester were analysed in the hexane upper phase by chromatography on alumina as described previously (Saari and Bredberg, 1990). The combined lower phases were acidified to pH 2–3 with 125 μl of 6 M HCl and then extracted with 2 × 3 ml of light petroleum. Two extractions of the acidified lower phase provided a 95% yield of non-esterified fatty acid, verified with [^{14}C]palmitate. After methylation with BF_3/methanol, fatty acid methyl esters were analysed with a Hewlett–Packard model 5890A gas chromatograph, by using a double-ramp temperature program from 45° to 240 °C and a QV-1701 capillary column for optimum separation.

RESULTS AND DISCUSSION

Exchange reactions of LRAT

Addition of retinol to buffered RPE microsomes results in an immediate burst of retinyl ester synthesis, followed by a rapid transition to a steady-state condition (plateau) in which the amount of retinyl ester does not appear to change (Saari and Bredberg, 1988). Previous studies had established that this CoA-independent reaction was due to LRAT (Saari and Bredberg, 1989). In this study the apparent steady state phase of the reaction was examined in more detail.

Several possible reasons for the abrupt halt in retinyl ester synthesis were considered. Retinol depletion was ruled out, as substrate levels of unesterified retinol remain at the plateau region of the progress curve (results not shown). Depletion of PC, the other substrate, has not occurred unless only a small fraction of the total PC can serve as a substrate for the reaction. Product inhibition by 2-acyl-lysoPC was not tested directly, since the propensity for acyl migration with this lysolipid (DeHaas and Van Deenen, 1965) would have made the results difficult to interpret. However, the more stable 1-acyl-lysoPC did not inhibit LRAT when added at concentrations up to 20 μM (results not shown). Product inhibition by retinyl ester can be ruled out, since addition of palmitoyl-CoA at the plateau results in further retinyl ester synthesis (Saari and Bredberg, 1988).

An experiment was designed to determine whether the lack of accumulation of retinyl ester at the steady state resulted from decreased retinyl ester synthesis. Non-radioactive retinol was incubated with microsomes for 20 min to allow the reaction to reach the plateau phase. Substrate amounts (5 μM) of [^{3}H]retinol were then added to the reaction mixture, and the specific radioactivities of retinol and retinyl esters were monitored for an additional 30 min. The results of the experiment, shown in Figure 1, demonstrate that [^{3}H]retinol is rapidly incorporated into retinyl ester. However, even though the amount of incorporation predicts a 30% increase in the amount of retinyl ester, there is little additional accumulation of retinyl ester as measured by spectral analysis. The specific radioactivities of [^{3}H]retinol and [^{3}H]retinyl ester reach the same value (Figure 1), a characteristic of a reaction at equilibrium. Thus it is apparent that retinyl ester synthesis continues during the plateau phase of the reaction and is opposed by another reaction that consumes it.

Two possibilities for the opposing reaction are a reversal of the LRAT reaction or hydrolysis of retinyl ester. We examined the latter possibility by using g.l.c. to determine the non-esterified fatty acid concentrations in samples of RPE microsomes that had been incubated for 20 min with and without [^{3}H]retinol. If retinyl ester hydrolysis were active during the plateau phase of the reaction, the amount of non-esterified fatty acid should be increased after incubation of microsomes with retinol. Details of the procedure used for extraction, methylation and analysis of

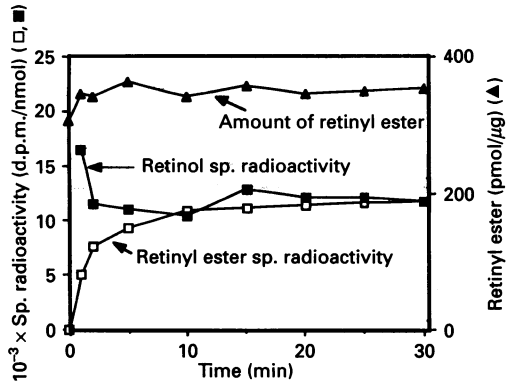

Figure 1 Exchange of all-*trans*-retinol with all-*trans*-retinyl ester

Retinyl ester synthesis was initiated by addition of unlabelled 5 μM all-*trans*-retinol to buffered RPE microsomes. After 20 min of reaction at 37 °C, 5 μM [^{3}H]all-*trans*-retinol (41 000 d.p.m./nmol) was added. BSA was present at 60 μM. The specific radioactivities of retinol (■) and retinyl ester (□) and the amount (▲) of retinyl ester are shown as a function of time after addition of [^{3}H]all-*trans*-retinol. The amount of retinyl ester was determined from its A_{320}.

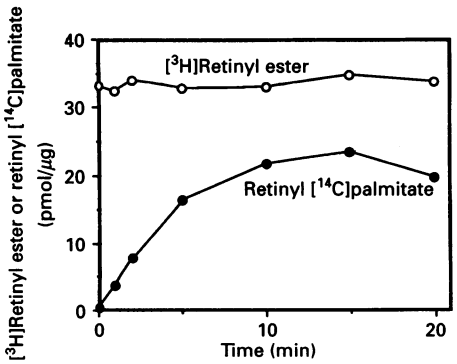

Figure 2 Exchange of palmitoyl-CoA with retinyl ester

[^{3}H]Retinol was added to microsomes at 80% of the amount determined to be esterified at the plateau of the progress curve. After 20 min of reaction at 37 °C, [^{14}C]palmitoyl-CoA (20 μM) was added. Retinyl ester was isolated and ^{3}H (○) and ^{14}C (●) were determined as a function of reaction time after addition of palmitoyl-CoA. The specific radioactivities of the retinyl esters differ, owing to dilution of the specific radioactivity of [^{14}C]palmitate by endogenous pools.

fatty acid methyl esters are provided in the Experimental section. During a 20 min incubation with retinol, 1345 pmol of retinyl ester was formed. The sample of microsomes incubated without retinol contained 788 pmol of palmitate, whereas the sample incubated with retinol contained 730 pmol of palmitate (averages of two determinations). The measured $V_{max.}$ of 30 nmol/min per μg of protein for incorporation of [^{3}H]retinol into retinyl ester (see below) predicts that virtually all of the retinyl ester would have turned over in 20 min, producing an excess of 1345 pmol of fatty acid in the sample incubated with retinol, 66% of which would be palmitate (Futterman and Andrews, 1964). Turnover of even half this amount would have been detectable over the background of palmitate present in the microsomal reaction mixture.

Published progress curves for esterification provide further evidence that retinyl ester hydrolysis is unlikely to be involved in generation of the steady state. Retinyl ester is stable for at least a 2 h period (see Figure 1; Saari and Bredberg, 1988) or decreases very slowly, even though retinol has been depleted by this time, a result incompatible with a retinyl ester hydrolase that matches the initial rate of retinyl ester esterification.

Although the rapid incorporation of [^{3}H]retinol into retinyl ester during the apparent stationary phase is strongly suggestive of a reaction at equilibrium, we have been unable to demonstrate reversibility by addition of substrates for the reverse reaction. Addition of retinyl [^{14}C]palmitate or retinyl [^{14}C]valerate after retinyl ester synthesis failed to produce significant labelling of PC. However, exogenous di[^{14}C]palmitoyl PC is a very poor substrate for the LRAT reaction with RPE microsomal preparations (Saari and Bredberg, 1988), suggesting that exogenous PCs do not readily equilibrate with microsomal lipids serving as substrates. It is also possible that high concentrations of PC in the local environment of the membrane drive the reaction in the direction of retinyl ester synthesis.

The lecithin:cholesterol acyltransferase reaction, which involves acyl transfer from the *sn*-2 position of PC to cholesterol, also displays exchange complexities. The reaction mechanism has been postulated to involve initial acyl transfer from PC to an enzyme seryl hydroxy group, followed by acyl migration to enzyme cysteinyl thiol groups and then to cholesterol, the ultimate acyl acceptor (Jauhianinen and Dolphin, 1986). Apparently only the last reaction is reversible, leading to accumulation of [^{3}H]cholesterol in the cholesterol pool after incubation of the enzyme with [^{3}H]cholesteryl [^{14}C]oleate, even though addition of excess lysoPC does not produce labelled PC (Sorci-Thomas et al., 1990). Although such a mechanism could explain some of the exchange properties observed here with LRAT, the complexity of the microsomal system precludes making a definite statement at present.

An exchange of [^{14}C]palmitate from [^{14}C]palmitoyl-CoA into retinyl palmitate could also be demonstrated. In this experiment [^{14}C]retinol was added to microsomes in an amount calculated to be nearly completely esterified (80% of the retinol esterified at the plateau in a control experiment). [^{14}C]Palmitoyl-CoA was then added, and ^{3}H and ^{14}C in retinyl palmitate were determined. As shown in Figure 2, [^{14}C]palmitate is incorporated into retinyl palmitate in the absence of a net accumulation of retinyl ester. The mechanism for this exchange is likely to involve acylation of lyso-PC by [^{14}C]palmitoyl-CoA, followed by PC participation in the LRAT reaction.

The specificity of retinol exchange was examined in more detail. The experiment involved microsomal synthesis of either all-*trans*- or 11-*cis*-retinyl ester as described for the experiment of Figure 1, followed by addition of either [^{3}H]11-*cis*- or [^{3}H]all-*trans*-retinol. The results, summarized in Figure 3, indicate that

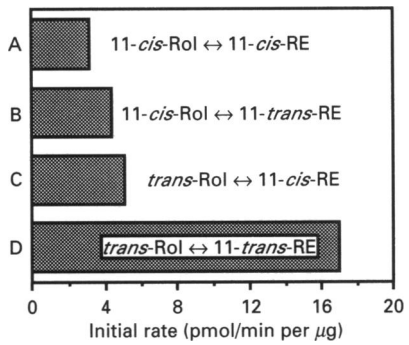

Figure 3 Specificity of exchange of retinols with retinyl ester

Reaction conditions are as described in the legend to Figure 1, except that two stereoisomers of retinol were employed. (**a**) Exchange of 11-*cis*-retinol with 11-*cis*-retinyl ester. (**b**) Exchange of 11-*cis*-retinol with all-*trans*-retinyl ester. (**c**) Exchange of all-*trans*-retinol with 11-*cis*-retinyl ester. (**d**) Exchange of all-*trans*-retinol with all-*trans*-retinyl ester. For determination of the initial exchange rates shown on the abscissa, 1- and 2-min assays were used. Abbreviations: Rol, retinol; RE, retinyl ester; ↔, exchange).

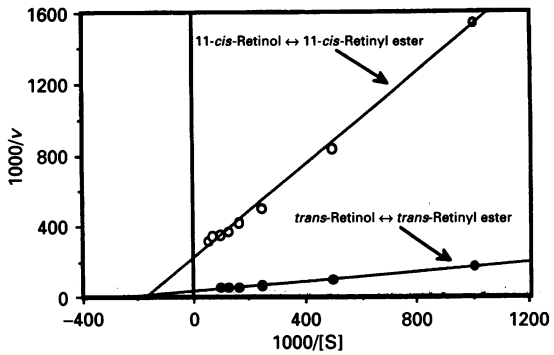

Figure 4 Kinetic constants characterizing retinoid exchange

Reaction conditions are as described in Figure 1, except that different concentrations of [^{3}H]all-*trans*-retinol were added to a reaction mixture containing all-*trans*-retinyl ester (●), or [^{3}H]11-*cis*-retinol to a reaction mixture containing 11-*cis*-retinyl ester (○). The results are shown as a double-reciprocal plot, where v = velocity (μM/min)) and [S] = substrate concentration (μM).

the fastest exchange was obtained with all-*trans*-retinol and all-*trans*-retinyl ester. Other possible exchanges are significantly slower. Kinetic constants for two of the exchanges were determined (Figure 4): exchange of all-*trans*-retinol with all-*trans*-retinyl ester, $K_m = 4.2\ \mu$M, $V_{max.} = 30.4$ nmol/min per mg of protein; exchange of 11-*cis*-retinol with 11-*cis*-retinyl ester, $K_m = 6.0\ \mu$M, $V_{max.} = 4.6$ nmol/min per mg of protein. The K_m values for the two isomers are similar to those reported previously for their esterification by LRAT (Saari and Bredberg, 1988), whereas the $V_{max.}$ values demonstrate an approx. 6-fold difference in the processing of the all-*trans* and 11-*cis* isomers that was not observed in the previous study of their esterification. These results are the first to demonstrate a difference in the processing of 11-*cis*- and all-*trans*-retinol associated with esterification, and may be important in understanding the metabolism of these two physiologically relevant isomers of vitamin A during dark adaptation.

It is not known if reversal of the LRAT reaction is of physiological significance. As a component of the vertebrate visual cycle, LRAT esterifies all-*trans*-retinol and thus provides substrate for the isomerization reaction (Deigner et al., 1989; Trehan et al., 1990). LRAT also esterifies 11-*cis*-retinol, the

product of this reaction, permitting a build-up of 11-*cis*-retinyl esters in the dark. Indeed, up to 60% of the accumulated retinyl esters can be of the 11-*cis* configuration (Bridges, 1976). Hydrolysis of 11-*cis*-retinyl ester, which must occur before it can be used for visual-pigment regeneration, is thought to involve retinyl ester hydrolases (Blaner et al., 1987; Mata et al., 1992). Mata et al. (1992) measured the stoichiometry of retinol and palmitate production from all-*trans*-retinyl palmitate and found essentially equal amounts of each, a result consistent with retinyl ester hydrolase activity and not reversal of LRAT. However, the conditions of their study were quite different from those employed here. In particular, it is unclear whether retinyl ester generated *in situ* by LRAT is equivalent to retinyl ester added to microsomes with respect to accessibility to enzymes. The observations reported in this paper suggest that reversal of the LRAT reaction could be a component in the mobilization of 11-*cis*-retinyl ester for visual-pigment renewal. Further studies are needed to determine whether the back reaction could provide a quantitatively significant amount of retinol. One of the goals of this study was to establish baseline conditions for study of the LRAT reaction; hence, minimal reaction conditions were employed. Other factors such as cellular retinol-binding protein ('CRBP') and cellular retinaldehyde-binding protein ('CRALBP') are likely to affect the LRAT reaction and, in particular, the processing of all-*trans*- and 11-*cis*-retinol isomers.

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REFERENCES

Blaner, W. S., Das, S. R., Gouras, P. and Flood, M. T. (1987) J. Biol. Chem. **262**, 53–58
Blomhoff, R., Green, M. H., Berg, T. and Norum, K. R. (1990) Science **250**, 399–404
Bridges, C. D. B. (1976) Exp. Eye Res. **22**, 435–455
DeHaas, G. H. and Van Deenen, L. L. M. (1965) Biochim. Biophys. Acta **106**, 315–325
Deigner, P. S., Law, W. C., Canada, F. J. and Rando, R. R. (1989) Science **244**, 968–971
Futterman, S. and Andrews, J. S. (1964) J. Biol. Chem. **239**, 81–84
Jauhianinen, M. and Dolphin, P. J. (1986) J. Biol. Chem. **261**, 7032–7043
MacDonald, P. N. and Ong, D. E. (1988) J. Biol. Chem. **263**, 12478–12482
Mata, N. L., Tsin, A. T. C. and Chambers, J. P. (1992) J. Biol. Chem. **267**, 9794–9799
Rando, R. R. (1991) Prog. Retinal Res. **10**, 161–178
Saari, J. C. (1990) Prog. Retinal Res. **9**, 363–381
Saari, J. C. and Bredberg, D. L. (1988) J. Biol. Chem. **263**, 8084–8090
Saari, J. C. and Bredberg, D. L. (1989) J. Biol. Chem. **264**, 8636–8640
Saari, J. C. and Bredberg, D. L. (1990) Methods Enzymol. **190**, 156–163
Sorci-Thomas, M., Babiak, J. and Rudel, L. L. (1990) J. Biol. Chem. **265**, 2665–2670
Trehan, A., Cañada, F. J. and Rando, R. R. (1990) Biochemistry **29**, 309–312
Yost, R. W., Harrison, E. H. and Ross, A. C. (1988) J. Biol. Chem. **263**, 18693–18701