Liposome-Mediated Enzymatic Synthesis of Phosphatidylcholine as an Approach to Self-Replicating Liposomes

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Abstract: The four enzymes of the salvage pathway for phosphatidylcholine synthesis sn-glycerol-3-phosphate acyltransferase, 1-acyl-sn-glycerol-3-phosphate acyltransferase, phosphatidate phosphatase, and cytidinediphosphocholine phosphocholinetransferase were simultaneously bound to soybean phosphatidylcholine liposomes. Evidence is presented that the entire enzyme chain is reconstituted in the liposomes, and that synthesis is preferentially localized in the proteliposomal membrane where the enzymes are present in an active bound form. Various phosphatidylcholines, differing in the alkyl group of the fatty acid moieties, can be synthesized in this way and incorporated into the host liposomes, with a corresponding change of the equilibrium size of the liposomes. The present system can then also be seen as a self-reproducing liposome, and the implication of this for chemical autopoiesis is briefly discussed.

Introduction

Chemical reactions occurring in the interior or at the interface of liposomes represent fascinating analogies with cellular reactions.[1,2] The analogy with cellular chemistry would be even stronger if enzymes were involved in the process. Proteoliposomes, i.e., enzyme-containing liposomes, may for example be seen as reconstituting a basic enzymatic cellular process.[3,4]

This paper describes the reconstitution of an enzymatic process: The four enzymes involved in the so-called salvage pathway synthesis[5] of phosphatidylcholine (PC) (Scheme I) are simultaneously inserted in liposomes, and the extent of PC production of this proteoliposomal system is studied.

The four enzymes are G3P-AT (sn-glycerol-3-phosphate acyltransferase), LPA-AT (1-acyl-sn-glycerol-3-phosphate acyltransferase), PA-P (phosphatidate phosphatase), and CDPC-PT (cytidinediphosphocholine phosphocholinetransferase). Their partial purification and basic chemistry are described in the literature.[6-9] To the best of our knowledge, the interaction of all these enzymes with each other or their interaction with vesicles or liposomes has not been reported yet. Thus, the present study offers a first example for the reconstitution of a PC-synthesizing machinery mediated through proteoliposomes.

Another aspect of this work is based on the use of proteoliposomes as a self-replicating system, where newly synthesized PC molecules are incorporated in the PC layers of the host liposomes. This will result in a growth of the total membrane surface, and possibly an increase in the population of the liposomes, i.e., in a self-replication process. Since this process is due to reactions taking place within the boundary of a geometrically closed structure, we can apply the notion of autopoiesis[10] as we have done in previous works dealing with the self-replication of micelles.[11,12] In the present paper, the aspects of self-replication and autopoiesis will be particularly emphasized in light of the comparison between micelles and liposomes.

Material and Methods

All solvents were obtained from Merck as p.A.-grade. Solid products were generally obtained from Fluka in the highest available degree of purity. Soybean PC was a gift from Hoffmann-La Roche. It contains less then 3% lysophosphatidylcholine and 0.5% phosphatidylethanolamine as judged by TLC (methanol/diethylether/toluene/cyclohexane/25% ammonia/sodium acetate (55:20:20:5:2:1 (v/v) + 2 g/L) as mobile phase on silica gel 60 F-254 (Merck)). Derivatives of coenzyme A were obtained from Sigma and [14]C- and ^{3}H-labeled substrates from Amersham.

Centrifugations in the low speed range up to 10000g were carried out on a Sorvall RC-5B Refrigerated Superspeed Centrifuge with a GSA-rotor from Du Pont. In the high speed range up to 100000g, a Centrikon

T-2070 with a TFT 70.38-rotor from Kontron was used.

Quasielastic light-scattering measurements were carried out at 25 °C and at a scattering angle of $\theta = 90°$ with a Malvern 4700 PS/MW that is equipped with a computer-driven goniometer and an argon ion laser (Coherent Innova 200). A z-average collective diffusion coefficient ($\langle D_c \rangle_z$) was obtained from a second-order cumulant analysis of the intensity autocorrelation function.[13] From $\langle D_c \rangle_z$ an apparent hydrodynamic radius ($R_h (=\langle R_h^{-1} \rangle_z^{-1})$) was calculated by using the Stokes-Einstein relation. A number of measurements were further analyzed by using a Laplace inversion method by means of an eigenfunction technique.[14] A modification of the original method of Ostrowsky et al.[15] was used.[16] Details are given elsewhere.[16]

The radioactivity of the spots on thin-layer chromatography plates was determined with a TLC Linear Analyzer Chromelec 101 from Numelec or a LB2820 from Berthold. Scintillation counting was carried out with a Tri-Carb 2200CA from Packard. Butyl-PBD (7 g/L) in toluene was used as the scintillation cocktail.

Microsomes were prepared following standard procedures.[17] Details are given elsewhere.[18] Partial purification of G3P-AT was carried out according to a modified method first described by Green et al.[6] Details for this preparation are given elsewhere.[18] Partial purification of LPA-AT was carried out according to a method described by Miki et al.[19]

(1) Fendler, J. S. *Acc. Chem. Res.* **1980**, *13*, 7-13.

(2) Fendler, J. S. *Membrane Mimetic Chemistry*; John Wiley & Sons: Chichester, 1982.

(3) Silvius, J. R.; Allen, T. M. *Biophys. J.* **1989**, *55*, 207-208.

(4) Green, P. R.; Bell, R. M. *J. Biol. Chem.* **1984**, *259*, 14688-14694.

(5) Stryer, L. *Biochemistry*; W. H. Freeman and Co.: San Francisco, 1975; p 482.

(6) Green, P. R.; Merrill, A. H., Jr.; Bell, R. M. *J. Biol. Chem.* **1981**, *256*, 11151-11159.

(7) Ichihara, K.; Asahi, T.; Fujii, S. *Eur. J. Biochem.* **1987**, *167*, 339-347.

(8) Caras, I.; Shapiro, B. *Biochim. Biophys. Acta* **1975**, *409*, 201-211.

(9) Kanoh, H.; Ohno, K. *Eur. J. Biochem.* **1976**, *66*, 201-210.

(10) Varela, F. G.; Maturana, H. R.; Uribe, R. *BioSystems* **1974**, *5*, 187-196.

(11) Luisi, P. L.; Varela, F. J. *Origins Life* **1989**, *19*, 633-643.

(12) Bachmann, P. A.; Walde, P.; Luisi, P. L.; Lang, J. *J. Am. Chem. Soc.* **1990**, *112*, 8200-8201. Bachmann, P. A.; Walde, P.; Luisi, P. L.; Lang, J. *J. Am. Chem. Soc.*, in press.

(13) Koppel, D. E. *J. Chem. Phys.* **1972**, *57*, 4814-4820.

(14) McWhirter, J. G.; Pike, E. R. *J. Phys. A: Math. Gen.* **1978**, *11*, 1729-1745.

(15) Ostrowsky, N.; Sornette, D.; Parker, P.; Pike, E. R. *Opt. Acta* **1981**, *28*, 1059-1070.

(16) Schurtenberger, P.; Schurtenberger, S. Manuscript in preparation.

(17) Kanoh, H.; Ohno, K. *Methods Enzymol.* **1981**, *71*, 536-546.

(18) Schmidli, P. K. Ph.D. Dissertation, ETH Nr. 9179, Zürich, 1990.

(19) Miki, Y.; Hosaka, K.; Yamashita, S.; Handa, H.; Numa, S. *Eur. J. Biochem.* **1977**, *81*, 433-441.

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Scheme I. Salvage Pathway for Phosphatidylcholine Synthesis
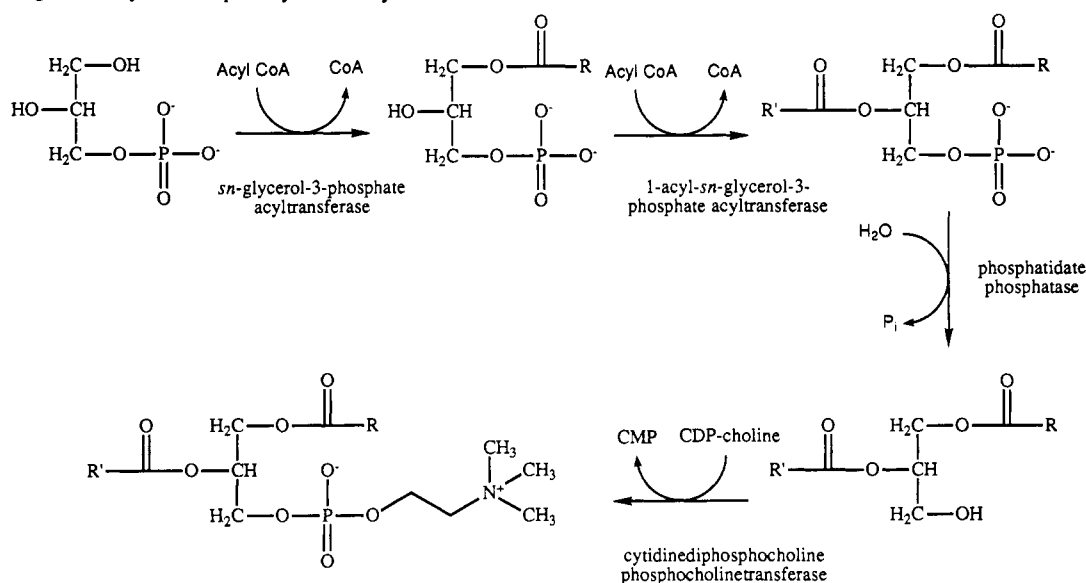
The purification of PA-P and CDPC-PT was carried out according to methods by Caras and Shapiro[8] and Kanoh and Ohno,[9] respectively.

Preparation of the Proteoliposomes. A mixed micellar solution of soybean PC and sodium deoxycholate (NaDC) ([PC] = 26 mM, [NaDC] = 33.7 mM) in Tris buffer (20 mM, pH = 7.4, [NaCl] = 140 mM) was prepared by the method of coprecipitation,[20] and 2 mg/mL of bovine serum albumin was added. This solution was then mixed with 1 volume of a 1.35% solution of sodium deoxycholate in Tris buffer (20 mM, pH = 7.4) that contained the partially purified enzymes. The ratio between the concentration of the enzymes and PC (w/w) was chosen to be 0.2.

Results and Discussion

Preparation and Characterization of the Proteoliposomes. The four enzymes were isolated from microsomes of pig liver as described in the Materials and Methods. It was not possible to isolate the four enzymes of the chain in one single procedure so that each of them had to be isolated with a particular procedure.

The partial purification of G3P-AT using dye–ligand and ion exchange chromatography resulted in a 32-fold increase of specific activity with respect to the microsomal preparation. In the case of the other enzymes, LPA-AT, PA-P, and CDPC-PT, a 5-fold increase of specific activity was obtained.

Enzyme-containing liposomes were prepared by means of dialysis. Sodium deoxycholate (NaDC) appeared to be a good surfactant as it was able to solubilize all enzymes without irreversible denaturation. Furthermore, liposomes with well-defined and reproducible physical properties can be prepared through dialysis of mixed micellar solutions of PC and NaDC. This preparation method permits to control the size of the liposomes through the ratio of surfactant to phospholipids before dialysis. The dialysis of a mixed micellar solution (initial molar ratio PC/NaDC = 0.4, weight ratio PC/total enzyme = 0.2) results in proteoliposomes with a mean hydrodynamic radius of R_h = 26 ± 0.2 nm. A small increase of the hydrodynamic radius for the enzyme-containing liposomes could thus be observed when compared to the enzyme-free liposomes, which have a hydrodynamic radius of 23 ± 0.2 nm (initial molar ratio PC/NaDc = 0.4). The analysis of the liposome solutions with dynamic light scattering showed that the resulting liposomes are quite monodisperse, with a normalized second cumulant of less than 0.07.

With the use of an average head group area of a = 0.64 nm^2 for PC molecules in a bilayer (see also below), a mean hydrodynamic radius of 26 nm corresponds to an aggregation number of approximately 1.3×10^3. Typically, at the end of the preparation, the ratio between PC and the sum of all proteins was 5, expressed in weight. Assuming as an approximate value of 100

(20) Small, D. M.; Penkett, S. A.; Chapman, D. *Biochim. Biophys. Acta* **1969**, *176*, 178–189.

Table I. Effect of the Different Types of Coenzyme A Fatty Acid Esters Used as Substrates on the Relative Activity of the G3P-AT[a]

type of the fatty acid	relative activity
C8:0	32
C16:0	100
C18:0	47
C18:1	22

[a] The protein concentration was 2 mg/mL and the soybean phosphatidylcholine concentration 13 mM in 70 mM sodium chloride, 20 mM Tris, pH 7.4 As substrate, sn-glycerol-3-phosphate was used in a concentration of 1 mg/mL.

Table II. Effect of Divalent Cations on the Relative Activity of G3P-AT and CDPC-PT[a]

	cation concentration				
	G3P-AT			CDP-PT	
cation	1 mM	5 mM	10 mM	10 mM	20 mM
Ca^{2+}	92	100	98	25	
Mg^{2+}	69	81	85	81	100
Mn^{2+}	55	71	71	66	65

[a] The protein concentration was 2 mg/mL and the soybean phosphatidylcholine concentration 13 mM in 70 mM sodium chloride, 20 mM Tris, pH 7.4. As substrates for G3P-AT, palmitoyl coenzyme A and sn-glycerol-3-phosphate were used in a concentration of 1 mg/mL each. For CDPC-PT, diglycerides from egg yolk and cytidinediphosphocholine were used in a concentration of 1 mg/mL each as well.

kD for the molecular weight for the enzymes, this means that we have a molar excess of PC to each of the proteins of about 600. On the basis of this molar ratio of PC to enzyme and the above-mentioned aggregation number, we conclude that a proteoliposome contains an average of 20 enzymes.

It is possible to show by gel chromatography on sepharose 2B that after this preparation all enzymes are indeed associated with the liposomes. The elution profile contains no low molecular weight component corresponding to free proteins.

Only the reconstituted enzymes in the proteoliposomes showed enzymatic activity. No activity was detected in any of the micellar solutions of the individually purified proteins. In general, the enzymatic properties in the proteoliposomes correspond to those in the microsomes. The partially purified G3P-AT has the same substrate specificity (Table I), and the influence of the type and concentration of bivalent cations on the enzymatic activity is the same (Table II). Figure 1 shows the pH–activity profile of G3P-AT: The optimal pH range is rather broad, with a maximal activity around pH = 7.4. In Figure 1, the pH–activity profile of CDP-PT is shown as well, whereas the influence of the cations on the activity is shown again in Table II. We thus conclude that

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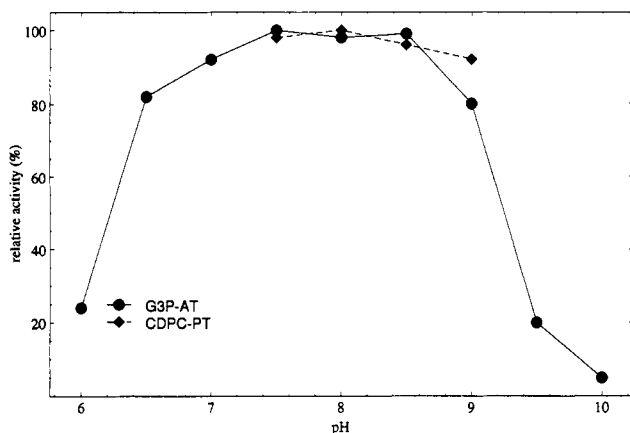*J. Am. Chem. Soc., Vol. 113, No. 21, 1991* 8129

Figure 1. Effect of pH on the relative activity of G3P-AT and CDPC-PT. The protein concentration was 2 mg/mL and the soybean phosphatidylcholine concentration 13 mM in 70 mM sodium chloride in both cases. As substrates for G3P-AT, palmitoyl coenzyme A and *sn*-glycerol-3-phosphate were used in a concentration of 1 mg/mL each. For CDPC-PT, diglycerides from egg yolk and cytidinediphosphocholine were used in a concentration of 1 mg/mL each as well.
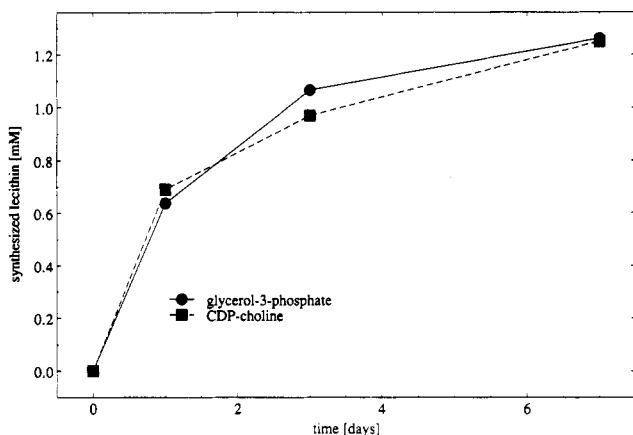

Figure 2. Proteoliposome-mediated synthesis of phosphatidylcholine. The protein concentration was 2 mg/mL and the initial soybean phosphatidylcholine concentration 13 mM in 70 mM sodium chloride, 20 mM Tris, pH 7.4. As substrates, *sn*-glycerol-3-phosphate, palmitoyl coenzyme A, oleoyl coenzyme A, and CDP-choline were used in a concentration of 10 mg/mL each. The synthesized phosphatidylcholine is mainly 1-palmitoyl-2-oleoylphosphatidylcholine.

the enzymatic activity is maintained in the proteoliposomes, and the general properties of the enzymes in the proteoliposomal form are not modified with respect to those in the microsomal form.

Synthesis of Phosphatidylcholine. The most important observation about the enzymatic activity concerns however the four-step synthesis of PC, starting from *sn*-glycerol-3-phosphate, as described in Scheme I.

The time progress of the reaction is given in Figure 2. One can see that the amount of newly synthesized PC is almost the same when analyzed as a function of the radioactive *sn*-glycerol-3-phosphate as substrate or when cytidinediphosphocholine is the radioactively labeled substrate. Since the other substrates for this reaction are palmitoyl and oleoyl coenzyme A, the produced PC is mainly 1-palmitoyl-2-oleoylphosphatidylcholine.[21,22] Figure 2 shows that the entire enzyme chain is successfully reconstituted in the proteoliposomes, and that they mediate the salvage pathway synthesis of PC.

Let us consider now the other aspect of this reaction, which has been mentioned in the Introduction with the keyword "self-

replication". The starting observation is again that although the substrates of the enzymatic chain reaction are water soluble, the product PC is an amphiphilic molecule that will be incorporated in the liposome bilayers. As a consequence, the total interface of the proteoliposomes is increasing during the reaction.

The question is now what the effect is of this on the physical state of the proteoliposomes. We expect a different effect according to whether the newly produced PC molecules have long (i.e., C16 or longer) or short (i.e., C10 or shorter) fatty acid residues in their alkyl moieties. The second case is particularly interesting, as one expects an increase of the spontaneous curvature of the liposomes. The incorporation of newly synthesized short-chain PC will thus result in a smaller equilibrium radius of the liposomes and a corresponding increase in the number of vesicles.

This expectation is based on the following geometrical considerations. We can estimate average values for the volume of the hydrocarbon chains, V_S, the headgroup area, a_S, and the critical length, l_S, for soybean PC from its known chemical composition and the measured equilibrium radius of the liposomes.[18,23,24] This leads to values of $V_S = 1.081$ nm^3, $l_S = 1.54$ nm, and $a_S = 0.64$ nm^2. If short-chain PC are incorporated, the average head group area does not change significantly (measured head group area for di-C6 PC is $a_6 = 0.66$ nm^2).[25] However, the average side chain volume V and length l decrease according to the following equations:

$$V = x_S V_S + (1 - x_S)V_6 \qquad l = x_S l_S + (1 - x_S)l_6$$

where V_S, V_6, l_S, and l_6 are the side chain volumes and lengths of soybean and di-C6 PC and x_S is the molar fraction of soybean PC in the bilayer. Using the new parameters V, l, and a in the expression of Israelachvilli[23] for the critical packing radius R_C of a vesicle, we find a smaller value for R_C for the two-component liposomes, which depends on x_S.

This theoretical prediction has been tested experimentally. Starting from soybean PC (average side chain length C16), di-hexanoylphosphatidylcholine (C6) has been synthesized by using the procedure described above with hexanoyl coenzyme A as substrate instead of palmitoyl and oleoyl coenzyme A. Light-scattering techniques have then been used to determine the size distribution of the proteoliposomes. As mentioned above, the initial proteoliposomes were monodisperse, with an average hydrodynamic radius of 26.0 ± 0.2 nm. After a 10% yield of the di-hexanoylphosphatidylcholine synthesis was reached (with respect to the initial molar concentration of soybean PC), the average radius decreased to 23 ± 0.3 nm, but no changes were observed in the width of the size distribution. These results clearly indicate that the size distribution of the liposomes relaxes after the incorporation of short-chain PC to new equilibrium values, in qualitative agreement with simple geometrical considerations, shown above.

Concluding Remarks

Some experimental aspects of this type of investigation may need optimization and further substantiation, which sets an objective in our future work. The work as it stands permits however already the following points to be made: (i) liposomes are suitable systems to reconstitute complex enzymatic chain reactions; (ii) the enzymatic activity is present only when the enzymes are bound to the liposomes, i.e., the reactions appear to be associated with the lipidic bilayer structure; (iii) liposomes are able to display self-replication; and (iv) the self-replicating liposomes can be seen as autopoietic systems.

Concerning the first point, there are two different aspects associated with the reconstitution: One is the possibility of modeling

(21) Yamashita, S.; Hosaka, K.; Numa, S. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 3490–3492.

(22) Yamashita, S.; Nakaya, N.; Miki, Y.; Numa, S. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 600–603.

(23) Israelachvili, J. N.; Mitchell, D. J.; Ninham, B. W. *Biochim. Biophys. Acta* **1977**, *470*, 185–20.

(24) Israelachvili, J. N. In *Physics of Amphiphiles: Micelles, Vesicles and Microemulsions*; Degiorgio, V., Corti, M., Eds.; North-Holland: Amsterdam, **1985**; pp 24–58.

(25) Tausk, R. J. M.; Karmiggelt, J.; Oudshoorn, C.; Overbeek, J. Th. G. *Biophys. Chem.* **1974**, *1*, 175–183.

complex cellular chain processes with a structure that, from the chemical point of view, is much simpler than a cell itself. The other aspect is the possibility of utilizing this approach for biotechnological purposes, i.e., for the production of PC.

The self-replication observed with the short-chain PC reveals the well-known extreme sensitivity of the supramolecular aggregate to the fine details of the chemical structure of the monomers: It also shows the versatility and tunability of these macromolecular complexes.

The self-replication is an important chemical process per se, as it provides a relatively simple synthetic system to mimick a basic cellular process. The relevance of this observation is made more stringent by the report that liposomes can be seen as prebiotic cells.[26,27]

To the best of our knowledge, no geometrically closed structures able to self-replicate have been thus far reported, except our micellar systems.[12] With respect to reverse micelles, liposomes offer the advantage of being closer to cells, regarding both their structure and the environment in which they operate. The fact that we are dealing with structures having a boundary that is itself constructed by the network of reactions taking place within the boundary itself permits to propose them as autopoietic units.[10,28] Autopoiesis can be defined as an operational definition of the living. The challenge in the liposome self-replicating system lies in the testing of this notion.

(26) Deamer, W. D.; Oro, J. *BioSystems* **1980**, *12*, 167–175.
(27) Morowitz, H. J.; Heinz, B.; Deamer, D. W. *Origins Life* **1988**, *18*, 281–287.

(28) Fleischacker, G. R. *BioSystems* **1988**, *22*, 37–49.

Mechanistic Studies on a Placental Aromatase Model Reaction

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Abstract: Aromatase is a cytochrome P-450 enzyme that converts androgens to estrogens via three successive oxidative reactions. The mechanism of the third step has previously been intensively studied, with no clear resolution. A leading theory for the third step proposes nucleophilic attack of the heme ferric peroxide species on the 19-aldehyde intermediate to produce a 19-hydroxy 19-ferric peroxide intermediate. We have shown previously that analogues of this intermediate failed to aromatize under nonenzymatic conditions. In this study, we prepared a 2,4-dien-3-ol analogue of the 19-aldehyde intermediate and showed that it reacted with HOOH to produce the corresponding estrogen derivative. Evidence has been accrued to suggest that this reaction, which we have called the aromatase model reaction, involves a 19-hydroxy 19-hydroperoxide intermediate. The model reaction was shown to be faithful to the actual aromatase-catalyzed reaction with regard to stoichiometric formic acid production, ^{18}O-incorporation patterns, and stereoselectivity for 1β-hydrogen removal. A kinetic analysis at 37 °C was also performed, and the reaction was demonstrated to be pseudo-first-order by using an excess of HOOH, and first-order with respect to HOOH at the concentrations studied. The effects of KOH, EDTA, and BHT on the reaction were also examined, and are discussed.

Placental aromatase is a cytochrome P-450 enzyme complex that catalyzes the conversion of steroidal androgens (testosterone (**1a**), androstenedione (**1b**)) to steroidal estrogens (estradiol (**4a**), estrone (**4b**)). The mechanism of aromatase has received widespread interest, in part because aromatase is a potential therapeutic target for selective lowering of estrogen levels in patients with estrogen-dependent tumors, including breast cancer.[1] Presumably elucidation of the aromatase mechanism could lead to the development of specific inhibitors. Furthermore, at a more fundamental level, the chemical reaction catalyzed by aromatase is complex and interesting and serves as a difficult challenge to mechanistic enzymologists.

Many aspects of the aromatase reaction have already been uncovered (see Figure 1). One human cytochrome P-450 protein[2] in conjunction with a nonspecific NADPH-dependent reductase is apparently responsible for effecting the entire conversion. Testosterone (**1a**), androstenedione (**1b**), and 16α-hydroxytesto-

sterone are all direct substrates for the enzyme with similar values for k_{cat} but different values of K_m. Two successive hydroxylations at the angular 19-methyl group lead to intermediate 19-hydroxy **2** and 19-oxo **3** compounds. Both reactions are thought to be classical cytochrome P-450 type hydroxylations, with 1 equiv of molecular oxygen and NADPH consumed in each step.[3] Both reactions are stereospecific, the first occurring with retention of configuration and the second involving loss of the 19 pro-R hydrogen. Interestingly, there is a significant kinetic isotope effect in the first step but none in the second.[4a] Only the first oxygen equivalent consumed is incorporated into the aldehyde, suggesting a stereospecific dehydration of the presumed *gem*-diol intermediate. Elegant studies, by Covey and co-workers, on the processing by aromatase of false substrates suggest that active-site groups direct this dehydration.[4b]

In the third and last step, another oxidation is thought to occur with consumption of a third equivalent of molecular oxygen and NADPH.[5] The 1β-hydrogen is lost to the aqueous medium[6] and

(1) (a) Brodie, A. M. H. *Biochem. Pharmacol.* **1985**, *34*, 3213. (b) Coombes, R. C.; Goss, P.; Dowsett, M.; Gazet, J.-C.; Brodie, A. *Lancet* **1984**, 1237. (c) Cole, P. A.; Robinson, C. H. *J. Med. Chem.* **1990**, *33*, 2933.
(2) (a) Ortiz de Montellano, P. R., Ed. *Cytochrome P-450: Structure, Mechanism, and Biochemistry*; Plenum: New York, 1986. (b) Means, G. D.; Mahendroo, M. S.; Corbin, C. J.; Mathis, J. M.; Powell, F. E.; Mendelson, C. R.; Simpson, E. R. *J. Biol. Chem.* **1989**, *264*, 19385. (c) Kellis, J. T.; Vickery, L. E. *J. Biol. Chem.* **1987**, *262*, 4413.

(3) Siiteri, P. K.; Thompson, E. A. *J. Steroid Biochem.* **1975**, *6*, 317.
(4) (a) Caspi, E.; Arunachalam, T.; Nelson, P. A. *J. Am. Chem. Soc.* **1986**, *108*, 1847 and references therein. (b) Beusen, D. D.; Carrell, H. L.; Covey, D. F. *Biochemistry* **1987**, *26*, 7833.
(5) Akhtar, M.; Calder, M. R.; Corina, D. L.; Wright, J. N. *Biochem. J.* **1982**, *201*, 569.