Tetrahedron Letters No. 18, pp 1555 - 1558, 1977. Pergamon Press. Printed in Great Britain.

INVESTIGATIONS OF SUBSTRATE SPECIFICITY OF SQUALENE SYNTHETASE

William N. Washburn* and Ronald Kow
Department of Chemistry, University of California, Berkeley, California, USA

(Received in USA 31 January 1977; received in UK for publication 28 March 1977)

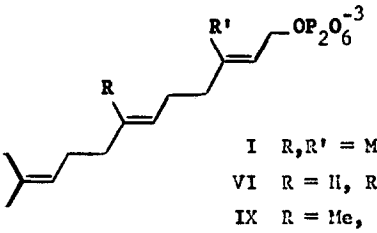
The substrate specificity of squalene synthetase has yet to be completely defined.[1,2] This enzyme complex will, in the presence of NADPH and Mg^{+2}, couple two farnesyl pyrophosphates (I) to form squalene[3] via an intermediate presqualene pyrophosphate.[4] We sought to probe this transformation by designing analogues of I which could only be partially metabolized as well as by delineating the minimum structural features of I necessary for substrate recognition. In contrast to farnesyl pyrophosphate synthetase,[5] we found this enzyme to be intolerant of structural modifications of I even when the changes occurred at the hydrocarbon tail of the molecule.

The following standard conditions were used for our incubations. The radiolabeled alcohols, prepared by LiB^3H_4 reduction of the corresponding aldehyde, were converted into pyrophosphates using Popjak's procedure.[6] The incubations were routinely carried out with 30 μg of the substrate pyrophosphate, 800mg of protein,[7] and 1.3mg of NADPH in 1ml in 0.05M phosphate buffer, pH 7.5, containing $MgCl_2$ (10mM), KF (10mM) and glycerol (30% by volume). Five minute incubations at 37° were used to compare the metabolic rates of the analogues relative to I. For product studies, larger amounts of protein in conjunction with longer incubation periods were employed. The incubations were quenched with ethanol and extracted with pet ether. Prior to scintillation counting, the residue was chromatographed on Eastman silica gel chromatogram sheets #13179 using 1% ether/pet ether.

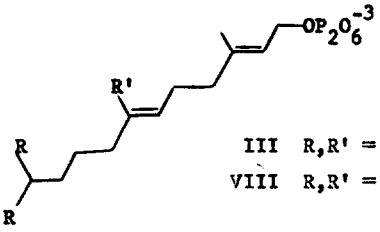
The discovery that E-6,7,10,11-tetrahydrofarnesyl pyrophosphate (II) was not metabolized either by itself or in the presence of I prompted an investigation to ascertain which of the missing double bonds were essential for substrate recognition. The 10,11-dihydrofarnesyl pyrophosphate III[9] was converted to a hydrocarbon[12] in 60% of the efficiency that I was converted to squalene. By C-GC-MS[13], the product was 2,3,22,23-tetrahydrosqualene (IV).[14]

In contrast to III, 6,7-dihydrofarnesyl pyrophosphate V[15] was not metabolized enzymatically either in the presence or absence of I. In an attempt to elucidate whether the role of the 6,7 double bond provided electronic stabilization of subsequent cations or induced the necessary conformation such that bonding of the tail could occur, we prepared the E,E-7-desmethylfarnesyl pyrophosphate (VI).[16] VI was converted enzymatically to a hydrocarbon at 10% of the metabolic rate of I. In the presence of equal amounts of unlabeled I, the conversion of VI to labeled

hydrocarbon diminished to 25% of the original metabolic rate of VI. C-GC-MS analysis revealed the structure of the product derived from VI to be a function of farnesyl pyrophosphate concentration. In the absence of I, the product was the symmetrical 6,19-didesmethylsqualene[18]; whereas in the presence of I, 6-desmethylsqualene (VII)[19] was formed.



I R,R' = Me
VI R = H, R' = Me
IX R = Me, R' = H



III R,R' = Me
VIII R,R' = H

The strong preference for the formation of the asymmetrical product suggested that one of the two binding sites for farnesyl pyrophosphate was discriminating against VI more strongly than the other. It was possible to confirm this hypothesis since the enzymatic coupling of the two farnesyl components proceeds with stereospecific loss of the pro S hydrogen from C_1 of one of the farnesyl components. Moreover, Porter, from a detailed kinetic study of squalene synthetase, had concluded that the two farnesyl pyrophosphates were bound sequentially and that the hydrogen was removed from the first farnesyl moiety bound at the active site.[22] Incubation of VI with 1,1-dideuterofarnesyl pyrophosphate in a ratio of 2:1 and 20:1 produced only squalene-d_3 and a 6-desmethylsqualene-d_1 in a ratio of 10:1 and 1:1. The failure to observe any 6-desmethylsqualene-d_2 established the difference in substrate binding specificities of the two sites.

As a rough approximation, the absence of a methyl at C_7 of I results in a discrimination against VI in favor of I by a factor of 20:1 at the second active site. The first binding site must discriminate more strongly by at least another factor of 10 against VI. It would appear likely that the C_6-C_7 double bond is essential for proper orientation of the chain so that binding can occur at each receptor site. The difference in metabolic rate with and without the C_7 methyl suggests that the methyl further enhances binding due to a specific hydrophobic interaction.

The importance of the remaining three methyl groups was examined. The additional 7 fold reduction in metabolic rate of E,E-3-methyl-2,6-undecadien-1-yl pyrophosphate (VIII)[23] relative to that of VI indicated that the gem dimethyls of the terminal isopropylidene group are important. Likewise, the C_3 methyl of I is essential. 3-Desmethylfarnesyl pyrophosphate (IX)[24] was inert both in the presence and absence of I. Under the conditions employed, products would have been detected if the conversion proceeded in greater than 2% of the efficiency of the conversion of I to squalene. Subsequent to the completion of our results, Ortiz de Montellano, using IX possessing much higher specific activity, reported detection

of the C_{29} hydrocarbon derived from the enzymatic coupling of IX and I in ~0.5% the efficiency of the conversion of I to squalene.

These results suggest that both of the active sites are quite specific. Our results in conjunction with those of Ortiz de Montellano permit a consistent picture with respect to the substrate specificity of the two binding sites for farnesyl pyrophosphate. The C_6-C_7 and C_{11}-C_{12} double bonds most likely serve to properly orient the molecule so that binding can occur. Each of the methyl groups, especially that at C_3 is essential for productive substrate binding to occur. Moreover, the first binding site responds more adversely to substrate analogues than the second regardless of the position of the structural modification. Due to the high specificity of the first active site, the failure of a substrate analogue to be metabolized equally at both sites does not allow distinction between alternative mechanistic pathways leading from presqualene pyrophosphate to squalene.

Acknowledgements: We thank Mr. Stephen Brown and Dr. Paul Philip for the GC-MS analysis and Petroleum Research Fund and National Institutes of Health grant #GM21578 for financial support. We thank Paul Ortiz de Montellano for exchange of results prior to publication.

References and Footnotes:

(1) At the outset of this project only structural analogues containing additional alkyl groups attached to C_{12} of the basic farnesyl unit had been reported. (a) A. Polito, G. Popjak and T. Parker, J. Biol. Chem., 247, 3464 (1972). (b) K. Ogura, T. Koyama and S. Seto, J. Amer. Chem. Soc., 94, 307 (1972). (c) A.A. Qureshi, F.J. Barnes, E.J. Semmler and J.W. Porter, J. Biol. Chem., 248, 2755 (1973).

(2) While this work was in progress two reports have appeared discussing the impact of structural modifications of the basic farnesyl unit. (a) P.R. Ortiz de Montellano, R. Castillo, W. Vinson and J.S. Wei, J. Amer. Chem. Soc., 98, 2018 (1976). (b) P.R. Ortiz de Montellano, R. Castillo, W. Vinson and J.S. Wei, J. Amer. Chem. Soc., 98, 3020 (1976).

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(4) F. Muscio, J.P. Carlson, L. Kuehl and H.C. Rilling, J. Biol. Chem., 249, 3746 (1974).

(5) T. Nishiso, K. Ogura and S. Seto, J. Amer. Chem. Soc., 94, 6849 (1972).

(6) J. Edmond, G. Popjak, S. Wong and V.P. Williams, J. Biol. Chem., 246, 6254 (1971). To determine if the sample was free of the monophosphorylated alcohol, the pyrophosphate was analyzed by tlc (i-PrOH, NH_3, H_2O; 6:3:1).

(7) Using a procedure reported by Porter[8] the protein was isolated from Bakers yeast by rupturing the cells by passage through a French pressure cell, centrifugation at 20,000 x g for 30 min., $(NH_4)_2SO_4$ induced fractionation (30-55% saturation) and dialysis.

(8) A.A. Qureshi, E. Beytia and J.W. Porter, J. Biol. Chem., 248, 1848 (1973).

(9) The alcohol corresponding to III was synthesized from 6-methyl-2-heptanone by the following steps: $Na(EtO)_2POCHCO_2Et$, AlH_3, PBr_3, $LiCH_2CCSiMe_3$,[10] $AgNO_3/H_2O$, BuLi,

$ClCO_2Et$, ϕSNa,[11] Me_2CuLi, tlc separation of the isomers (multiple developments using $CCl_4/CHCl_3$ in a ratio of 2:1), AlH_3. The specific activity of III was 0.17 Ci/mole.

(10) E.J. Corey and H.A. Kirst, Tetrahedron Lett., 5041 (1968).

(11) S. Kobagashi and T. Mukaiyama, Chem. Lett., 1425 (1974).

(12) To establish that the hydrocarbon was a squalene derivative, the incubation product was ozonized at -75°, submitted to oxidative workup and followed by a Jones oxidation. The product after dilution with succinic acid was recrystallized to constant activity.

(13) Computerized-gas chromatography-mass spectrometry (C-GC-MS) analysis was performed using a Dupont 491-2 double focusing mass spectometer coupled directly to a Varian Aerograph 204 gas chromatograph. Separations of the hydrocarbons were achieved by heating a 6.6m x 0.7mm glass capillary column packed with 3% Dexsil 300 on Gas Chrom Q from 200°-280° at 6°/min. A Dupont 21-094 data system continually acquired data throughout the analysis.

(14) Mass spectrum of IV at 50ev ionization source. m/e (%relative intensity): 414(10.1), 275(3.6); parent, p-139.

(15) The alcohol of V was prepared from 6-methylhept-5-en-2-one via the same procedure previously employed,[9] except for an Li/NH_3 reduction of ethyl 3,7-dimethyl-2,6-octadienonate. The specific activity of V was 0.12 Ci/mole.

(16) The tritiated alcohol of VI was prepared from 3,3-dimethylallyl chloride as follows: $LiCH_2CCSiMe_3$, $AgNO_3/H_2O$, BuLi, $AlEt_2Cl$,[17] MVK, $NaBH_4$, Li/NH_3, H_2CrO_4, T_2O/Et_3N, $Na(EtO)_2POCHCO_2Et$, tlc separation of isomers, AlH_3. The specific activity of VI was 0.18 Ci/mole.

(17) J. Hooz and R.B. Layton, J. Amer. Chem. Soc., 93, 7320 (1971).

(18) M/e (% relative intensity): 382(1.6), 339(2.9), 313(1.9), 271(0.5), 259(1.7). parent, p-43, p-69, p-43-68, p-69-54.

(19) M/e (% relative intensity): 396(2.8), 353(1.3), 327(2.0), 285(1.14), 273(1.62), 259(2.33). parent, p-43, p-69, p-43-68, p-69-54, p-69-68.
The structure of VII was confirmed by independent synthesis entailing treatment of farnesyl bromide with ϕSNa, nBuLi,[20] 7-desmethylfarnesyl bromide, $Li/EtNH_2$.[21]

(20) J.F. Biellmann and J.B. Ducep, Tetrahedron, 27, 5861 (1972).

(21) E.E. van Tamelen, P. McCurry and U. Huber, Proc. Nat. Acad. Sci. USA, 68, 1294 (1971).

(22) E. Beytia, A.A. Qureshi and J.W. Porter, J. Biol. Chem., 248, 1856 (1973).

(23) The alcohol corresponding to VIII was prepared by reacting 1-hexynyldiethylaluminum with MVK, $NaBH_4$, Li/NH_3, CrO_3/Pyr_2, T_2O/Et_3N, $Na(EtO)_2POCHCO_2Et$, tlc separation of the isomers, AlH_3. The specific activity of VIII was 0.03 Ci/mole.

(24) E.J. Corey, P.R. Ortiz de Montellano and H. Yamamoto, J. Amer. Chem. Soc., 90, 6254 (1968). The specific activity of IX was 0.02 Ci/mole.