Stereochemical Course of Hydrogen Transfer Catalysed by the Enoyl Reductase Enzyme of the Yeast Fatty Acid Synthetase

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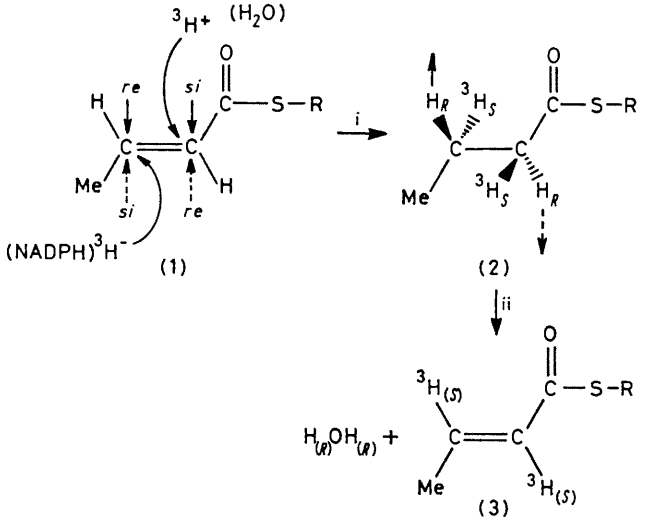
Summary Reduction of the *trans*-2-enoyl intermediate (**1**) to give the saturated alkyl thioester (**2**) in fatty acid biosynthesis occurs by means of an *anti*-addition of hydrogen to the *pro*-2*S* and *pro*-3*S* position of the carbons forming the double bond.

THE stereochemical course of three reactions involved in the biosynthesis *de novo* of long-chain fatty acids, namely the acetyl-CoA carboxylase, the β-ketoalkyl thioester synthase, and the hydroxyalkyl thioester dehydrase, has been investigated previously in this laboratory.[1] The enoyl reductase is the enzyme responsible for catalysing the final reaction in the cycle of condensation–reduction–dehydration–reduction that results in the lengthening of the fatty alkyl chain by one $-CH_2CH_2-$ unit at each turn of the cycle. It is also the last enzyme involved in this sequence of reactions whose stereochemical mechanism has not been elucidated.



SCHEME. Enzymes: i, enoyl reductase; ii, butyryl–CoA dehydrogenase.

This reaction and the basis of the experimental approach to define the stereochemical course of the reaction are illustrated in the Scheme. It results in the reduction of the *trans*-2-enoyl intermediate to the saturated derivative *via* an NADPH-dependent reaction in which a hydride ion from the *pro*-4*R* position of NADPH is transferred to C-3,[2] the reductant for C-2 being a proton derived from the water of the reaction medium.[3] Thus, the use of tritiated NADPH will result in a saturated alkyl thioester labelled with ^3H at C-3, whereas unlabelled NADPH and tritiated water during incubation will result in ^3H incorporation at C-2. Assuming that these reactions are stereospecific, the ^3H label on C-2 and C-3 will be either in the *pro*-*S* or the *pro*-*R* position; thus, there are four possibilities for the labelling pattern from the above experiments. These correspond to the stereochemical conformations described as (2*R*,3*R*), (2*S*,3*S*), (2*R*,3*S*), and (2*S*,3*R*) which would result, respectively, from *re–re*, *si–si*, *re–si*, and *si–re* additions of hydrogen at C-2 and C-3, according to the terminology of Hanson.[4] If *trans*-but-2-enoyl-*N*-acetylcysteamine (crotonyl-*N*-acetylcysteamine) is used as the substrate for this reduction, the product carrying this labelling pattern will be butyryl-*N*-acetylcysteamine. In order to determine the stereochemical position of the ^3H label in the butyric acid derived from the thioester product, use was made of the known stereochemistry of the reaction catalysed by butyryl–CoA dehydrogenase from pig liver. This enzyme has been shown to catalyse the *anti* elimination of the *pro*-2*R* and *pro*-3*R* hydrogens during oxidation to *trans*-crotonyl–CoA.[5,6] Thus, if butyric acid specimens, stereospecifically labelled with ^3H at either C-2 or C-3, are considered, stereochemical assignment of this label can be made by subjecting the acids (as CoA thioesters) to the above enzymic oxidation and subsequently determining whether ^3H is lost into the water of the medium or retained in the crotonyl–CoA produced.

Specimens of butyryl-*N*-acetylcysteamine (**2**; R = Me-$CONHCH_2CH_2-$) labelled either at C-3 or C-2 were obtained by incubating the unlabelled crotonyl thioester (**1**) with (4*RS*)-[4-^3H]NADPH[7] (1·6 Ci/mol) or with tritiated water (7·2 Ci/mol) in the presence of purified fatty acid synthetase from yeast. The labelled butyric acid was isolated from the product, purified, then mixed with carrier and [1-^{14}C]-butyric acid prior to synthesis of the CoA thioester *via* the mixed anhydride.[8] Those doubly labelled butyryl–CoA specimens (**2**; R = CoA), were purified by ion exchange chromatography,[1] then re-oxidised to crotonyl–CoA (**3**; R = CoA), using butyryl–CoA dehydrogenase from pig liver, essentially as described by Hoskins.[9] The acyl–CoA fraction, containing unchanged butyryl–CoA substrate plus crotonyl–CoA product, was purified by ion exchange chromatography and then subjected to complete alkaline hydrolysis (1M KOH, 2 h, 95 °C) to release the acids. These conditions were required to obtain effective cleavage of the crotonyl thioester bond, but the product released from crotonyl–CoA by this treatment was found to be 3-hydroxybutyric acid. As no loss of ^3H accompanied this hydration, the isotope ratio of the crotonyl–CoA product was subsequently determined as 3-hydroxybutyrate. The butyric and hydroxybutyric acids were separated by partition chromatography on Amberlite CG-120,[10] then further characterised *via* their crystalline *p*-bromophenacyl ester derivatives. In addition, (2*RS*)-[2-$^3H_1$]butyric acid and (3*R*)-[3-$^3H_1$]butyric acid were synthesised chemically and were also subjected, as their CoA esters, to the enzymic oxidation in order to check the activity of the dehydrogenase.

TABLE

Butyryl-CoA substrate			Crotonyl-CoA product (as 3-hydroxybutyrate *p*-bromophenacyl ester)	
^3H Labelling pattern	Origin	$^3H/^{14}C$ ratio	$^3H/^{14}C$ ratio	% ^3H retention
[3-^{3}H]	FAS	1 21	0 89	73 6
		1 21	0 80	66 1
(3*R*)-[3-^{3}H]	Synthetic	3 07	1 03	33 6
[2-^{3}H]	FAS	3 29	2 53	76 9
		3 29	2 62	79 6
(2*RS*)-[2-^{3}H]	Synthetic	4 09	1 99	48 5

The Table shows the isotope ratios of the substrate and product for the four butyryl–CoA specimens used in this work, with the ^3H content of the products expressed as a percentage of that originally present in the substrate It is seen that the product from the [3-^{3}H]butyrate derived from the fatty acid synthetase (FAS) retained an average of 69·8% of the original ^3H, indicating that this label was inserted into the *pro*-3*S* position during the enoyl reductase reaction The chemically synthesised (3*R*)-[3-^{3}H] standard on the other hand lost the majority (66 4%) of its original ^3H, as was expected [5,6] The non-ideal figures obtained using the [3-^{3}H]-substrates were unexpected and we do not know whether they represent racemisation of the label during preparation or on exposure to the dehydrogenase, or reflect the fact that the dehydrogenase may not be totally stereoselective for removal of the *pro*-3*R* hydrogen [5,6]

The product from the [2-^{3}H]butyrate derived from the FAS retained an average of 78 3% of the original ^3H which was, therefore, in the *pro*-2*S* position The 20% racemisation apparently present in this substrate is not unexpected in view of earlier predictions[1] concerning the nature of the 'post-malonate' proton exchange that was shown to occur during fatty acid biosynthesis The synthetic (2*RS*)-[2-^{3}H]-substrate, as expected, retained approximately half of the original ^3H on oxidation to crotonate

Thus, the mechanism of the enoyl reductase has been shown to involve an *anti* addition of hydrogen *via* a 2-*si*, 3-*si* attack on the double bond, as is shown in the Scheme

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