On the Stereospecificity of the Coenzyme B_{12}-Dependent Isobutyryl-CoA Mutase Reaction

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Received July 19, 1995®

Abstract: The stereospecificity of the reversible conversion of isobutyryl-CoA into *n*-butyryl-CoA, catalyzed by isobutyryl-CoA mutase from *Streptomyces cinnamonensis*, has been investigated by following the turnover of (2*S*)- and (2*R*)-[3-^{13}C] and [2-^{2}H]isobutyryl-CoA into labeled *n*-butyryl-CoAs by NMR methods. Using an enzyme extract containing the mutase, it was shown that (2*R*)-[3-^{13}C]isobutyryl-CoA is converted predominantly into [4-^{13}C]- and also at a lower rate into [2-^{13}C]*n*-butyryl-CoA. In a complementary experiment, it was shown that (2*S*)-[3-^{13}C]-isobutyryl-CoA is transformed rapidly into [2-^{13}C]- and more slowly into [4-^{13}C]*n*-butyryl-CoA. When [2-^{2}H]-isobutyryl-CoA was transformed on the enzyme both (3*R*)- and (3*S*)-[3-^{2}H$_1$]*n*-butyryl-CoAs were formed, with the (3*R*) isotopomer in excess. These results reveal a preferred stereochemical course at C2 of isobutyryl-CoA during the rearrangement which is retention, analogous to that observed for the methylmalonyl-CoA mutase reaction. However, the results clearly reveal a loss of stereocontrol during the isobutyryl-CoA mutase reaction, since the products of a formal inversion at C2 are also observed. This has been rationalized here by assuming that the substrate, isobutyryl-CoA, may bind at the active site and react from two different starting conformations, one of which is preferred, and/or that the enzyme loses strict control over the transformation or conformation of bound radical intermediates. The use of gradient enhanced inverse detected heteronuclear 2D ^{1}H−^{13}C NMR spectroscopy for monitoring the turnover of (2*S*)-[3-^{13}C]isobutyryl-CoA directly in the NMR tube is also described. This sensitive analytical method should be widely applicable for the direct monitoring of enzymic reactions using ^{13}C labeled substrates in the presence of large amounts of protium-containing buffer components.

Introduction

Isobutyryl-CoA mutase (ICM) (EC 5.4.99.13) is a coenzyme B_{12}-dependent enzyme that catalyzes the reversible interconversion of *n*- and isobutyryl-CoA and appears to be widely distributed in species of the Gram positive bacteria *Streptomyces*. This rearrangement was first detected during biosynthetic feeding experiments with shake cultures of macrolide and polyether antibiotic-producing streptomycetes, through the incorporation of ^{13}C labeled isobutyrate or valine into the *n*-butyrate unit in the macrolide aglycon of tylosin[1] and in the polyethers monensin A[2] and lasalocid A[3] . Later, ICM was detected in cell-free extracts of the monensin producer *Streptomyces cinnamonensis*, where it was also shown that isobutyryl- and *n*-butyrylcarba(dethia)-coenzyme A analogues are excellent substrates for the enzyme.[4] In this paper, we report the results of stereochemical studies with ICM from *S. cinnamonensis*,

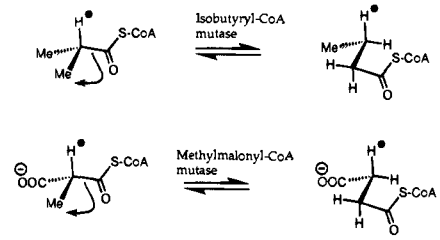

Figure 1. The reactions catalyzed by MCM and ICM.

which reveal a partial loss of stereochemical control over the transformation of isobutyryl-CoA into *n*-butyryl-CoA at the active site of this enzyme.

The reaction catalyzed by ICM is very similar to that of the well-known and widely distributed coenzyme B_{12}-dependent methylmalonyl-CoA mutase (MCM) (Figure 1). The rearrangement of isobutyryl-CoA is not catalyzed by MCM with a slack substrate specificity, as shown by cloning and expression[5] of the MCM gene from *S. cinnamonensis*. The MCM from this organism was shown to be closely related in primary sequence to that from *Propionibacterium shermanii*,[6] comprising a heterodimer with subunits of ≈ 65 kDa and ≈ 80 kDa. Like the *P. shermanii* enzyme,[4] the *S. cinnamonensis* MCM does not catalyze the interconversion of *n*- and isobutyryl-CoA at a measurable rate[5]. More recently, the ICM has been purified from *S. cinnamonensis* cell extracts and sequencing of internal tryptic peptides has revealed a close similarity in primary sequence to the corresponding regions of the MCM from this

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® Abstract published in *Advance ACS Abstracts*, October 15, 1995.

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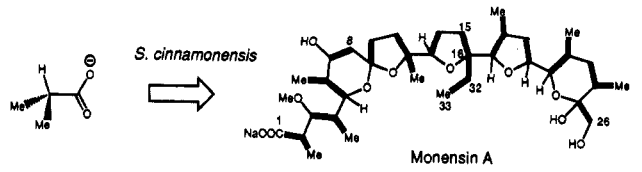
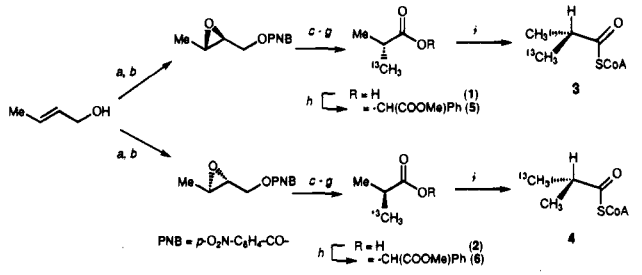


Figure 2. Incorporation of isobutyrate into the *n*-butyrate subunit (C15–C16–C32–C33) in monensin A.

Scheme 1[a]



[a] Reagents: *a*, Sharpless epoxidation;[11] *b*, PNB-Cl, Et_3N; *c*, KOH, MeOH; *d*, *t*-$BuMe_2SiCl$, Et_3N; *e*, $^{13}CH_3CuPCy_2Li$; *f*, *n*-Bu_4NF, THF; *g*, $KMnO_4$, $NaIO_4$; *h*, (+)-methyl mandelate, DCC, DMAP; *i*, $(Imid)_2CO$, CoA-SH.

organism, suggesting a close evolutionary relationship between these two mutases.[7]

The stereochemical course of the ICM reaction was deduced earlier[8] by incorporating various isotopomers of isobutyrate into the *n*-butyrate unit (i.e., C15–C16–C32–C33) in monensin A, in whole cell cultures of *S. cinnamonensis*. For example, sodium (2*S*)-[3-^{13}C]isobutyrate gave a fourfold enrichment of ^{13}C at C16 in monensin A, the position formally derived from C2 of *n*-butyrate (Figure 2). On the other hand, [2-^{2}H]-isobutyrate enriched C32, as expected, but the deuterium was distributed 85:15 between the pro-*R* and pro-*S* positions, respectively. These and other results[8] indicated the *predominant* migration of C1 of isobutyrate to the 2-pro-*S* methyl group and its replacement by a hydrogen atom with overall retention of configuration. The stereochemical course of this rearrangement is then comparable to that catalyzed by MCM[9,10] (Figure 1). However, the 85:15 distribution of deuterium between the diastereotopic centers at C32 observed in monensin A biosynthesized from [2-^{2}H]isobutyrate, suggested that the ICM reaction may not occur stereospecifically. With partially purified enzyme available this aspect of the ICM reaction has now been re-examined in vitro, by following the conversion of (2*S*)- and (2*R*)-[3-^{13}C] as well as [2-^{2}H] isobutyryl-CoA into labeled *n*-butyryl-CoAs, and the results are reported below.

Results

Synthesis. The method used to prepare sodium (2*S*)- and (2*R*)-[3-^{13}C]isobutyrate was a modification of that reported earlier.[8] Advantage was taken of an optimized *Sharpless* epoxidation of crotyl alcohol, utilizing molecular sieves and *in situ* derivatization to yield the corresponding epoxides as *p*-nitrobenzoate esters (Scheme 1), which can be recrystallized to >98% optical purity.[11] The epoxide moieties were opened efficiently in a later step with a mixed cuprate prepared from $^{13}CH_3Li$, $CuBr{\cdot}SMe_2$, and dicyclohexylphosphine[12] in 64% yield. The products were oxidized to their respectively labeled isobutyrates **1** and **2** and then converted to the coenzyme A thioesters **3** and **4**. The optical purity (96% ee) of each ^{13}C labeled isobutyrate was assayed by ^{13}C NMR after conversion to the (+)-mandelate derivatives **5** and **6**, as described earlier.[8]

Enzyme Assays. An assay for the ICM reaction has been described,[5] in which the products are quantified by GC after hydrolysis of the thioesters to the free carboxylic acids. In earlier studies, using isobutyrylcarba(dethia)-CoA as substrate, which is stable to hydrolysis, the equilibrium ratio of iso- and *n*-butyrylcarba(dethia)-CoA determined by NMR methods[4] was 57:43. Preliminary studies[7] indicate that similar equilibrium ratios can be achieved for the rearrangement of iso- to *n*-butyryl-CoA (data not presented). In the large scale assays performed here, using milligram amounts of substrate and a partially purified enzyme from *S. cinnamonensis*, conversions of iso-butyryl-CoA into *n*-butyryl-CoA in the range 20–30% at 30 °C were typically observed. The approach to equilibrium was hindered, however, by the limited half-life of the mutase ($\approx$ 60 min) at 30 °C in the enzyme extract and by the presence of a competing thioesterase activity that promotes hydrolysis of both iso- and *n*-butyryl-CoA; the free acids are not substrates for the mutase. Thioesterase-free ICM could not be obtained in yields high enough for the preparative-scale turnover of substrate required in these studies. The thioesterase was not inhibited by addition of phenylmethanesulfonyl fluoride, diisopropyl fluorophosphate, or iodoacetamide. In the absence of added coenzyme B_{12}, no conversion of iso- to *n*-butyryl-CoA is observed.

Turnover of [3-^{13}C]isobutyryl-CoA. (2*R*)-[3-^{13}C]Iso-butyryl-CoA (**4**) was incubated with the protein fraction containing ICM, and 25% was transformed to *n*-butyrate after $\approx$ 3 h, according to the GC assay. After hydrolyzing the remaining thioesters, the *n*- and isobutyrates were extracted, converted into *p*-bromophenacyl esters, separated by TLC, and then examined by proton decoupled inverse gated (5 s relaxation delay) ^{13}C NMR. As expected,[8] the methyl group in *p*-bromophenacyl *n*-butyrate was strongly enriched ($\approx$67%) in ^{13}C; however, a significant amount of ^{13}C label ($\approx$33%) was also detected at C2. Thus a partial scrambling of the label has occurred between the C2 and C4 positions of *n*-butyrate. Upon repeating this experiment with different incubation times, the enrichment at C4 was always greater than at C2, but that at C2 increased with longer incubation times. In addition, the *p*-bromophenacyl [3-^{13}C]isobutyrate recovered from the 3 h incubation was hydrolyzed, and the free acid was converted into the (+)-mandelate ester (see **5** and **6**, Scheme 1). ^{13}C NMR analysis showed that the ^{13}C label was now distributed 83:17 between (2*R*)- and (2*S*)-[3-^{13}C]isobutyrate. On the other hand, an identical 3 h incubation with (2*S*)-[3-^{13}C]isobutyrate (**3**), except that no coenzyme B_{12} was added, led to no conversion to *n*-butyryl-CoA, and the stereoisomeric purity of the recovered isobutyrate when assayed in the same way was unchanged (96% ee). This demonstrates clearly that the loss of stereochemical purity is caused by a coenzyme B_{12}-dependent enzyme.

When (2*S*)-[3-^{13}C]isobutyryl-CoA (**3**) was incubated with the enzyme and coenzyme B_{12}, a complementary result was obtained, with a greater enrichment in *n*-butyrate at C2, rather than at C4. For example, two identical incubations were performed with enzyme and (2*S*)-[3-^{13}C]isobutyryl-CoA (**3**), and one was interrupted at 10 min and the second at 60 min, by addition of NaOH. In the former experiment, $\approx$10%, and in the latter $\approx$25% turnover to *n*-butyryl-CoA occurred, as

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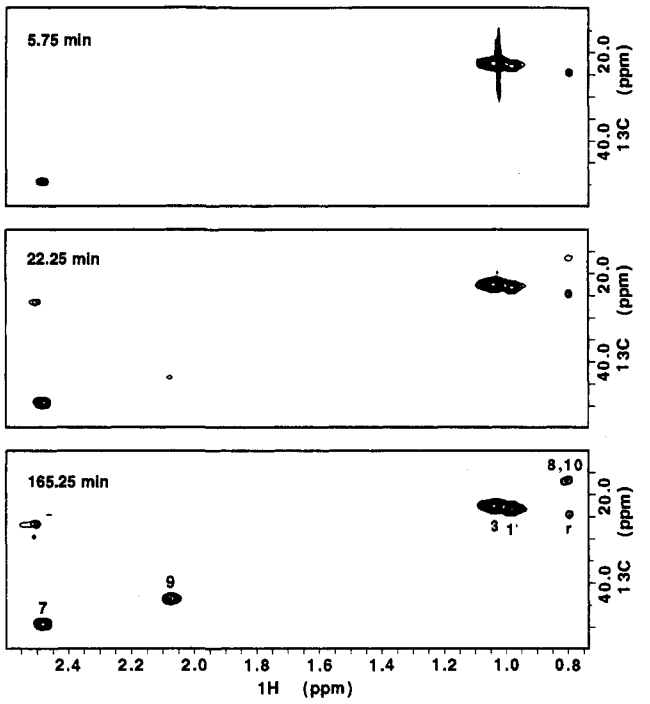

Figure 3. Gradient enhanced inverse 2D $^{1}H-^{13}C$ correlation spectra acquired during turnover of (2*S*)-[3-^{13}C]isobutyryl-CoA (**3**) in the *S. cinnamonensis* enzyme extract (see text). The elapsed reaction time at the midpoint of the acquisition is indicated in the upper left of each spectrum. The peaks are assigned to the products shown in Figure-4. The peak labeled "**r**" is the natural abundance ^{13}C signal of one of the geminal methyl groups in coenzyme A (the second does not appear in the region shown) and serves as an internal reference. The signal labeled with an asterisk is a baseline artefact (see Experimental Section for details).

determined by GC assay. ^{13}C NMR analysis of the isolated *n*-butyrates as their *p*-bromophenacyl esters showed a 3% enrichment of ^{13}C at C4 in the 10 min product but a 19% enrichment at C4 in the 60 min product. In both cases, the majority of the ^{13}C label was located at C2.

2D-NMR. To follow the conversion of (2*S*)-[3-^{13}C]isobutyryl-CoA (**3**) in the enzyme extract directly by NMR, gradient enhanced inverse detected heteronuclear 2D $^{1}H-^{13}C$ correlation experiments[13] were performed. These allow the rapid and selective detection of protons attached to ^{13}C; when using ^{13}C enriched substrate, all signals arising from nonlabeled centers, including those from buffer components, such as water, Tris, and glycerol, are effectively suppressed. Thus the labeled substrate (11 μmol) was added to protein (19 mg) and coenzyme B_{12} (0.25 μmol) in buffer A (0.45 mL) just prior to the acquisition of the first 2D spectrum. Each 2D $^{1}H-^{13}C$ shift correlation spectrum was accumulated in 5.5 min, thus allowing the acquisition of 30 spectra over an $\approx$3 h period. Three of the resulting spectra are shown in Figure 3, which give information about the fate of the substrate after 5.75, 22.25, and 165.25 min. The reaction products are depicted in Figure 4, and the time courses of their formation are shown in Figure 5. At 5.75 min signals from both [2-^{13}C]*n*-butyryl-CoA (**7**) and (2*S*)-[3-^{13}C]isobutyrate (**1**) as well as from (2*S*)-[3-^{13}C]isobutyryl-CoA (**3**) are apparent. By 165.25 min a substantial portion of the coenzyme A thioesters have been hydrolyzed to **1** and **9**, and [4-^{13}C]*n*-butyryl-CoA (**8**) has accumulated to a significant extent. (2*R*)-[3-^{13}C]isobutyryl-CoA (**4**) could not be detected since its ^{13}C-resonance is masked by that from **3**. The time course of the production of **8** + **10** are shown in Figure 5.

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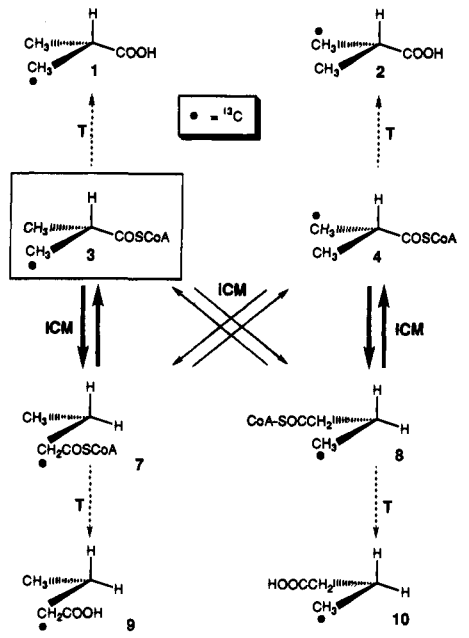


Figure 4. Transformation of (2*S*)-[3-^{13}C]isobutyryl-CoA (**3**) as monitored by 2D NMR (see Figure-3). Formation of **7** is faster than of **8** (Figure-5), catalyzed by **ICM**, as indicated by the bold and thin arrows, respectively. Reactions catalyzed by a thioesterase are indicated with a broken arrow and a **T**. The dot (•) indicates ^{13}C-label.

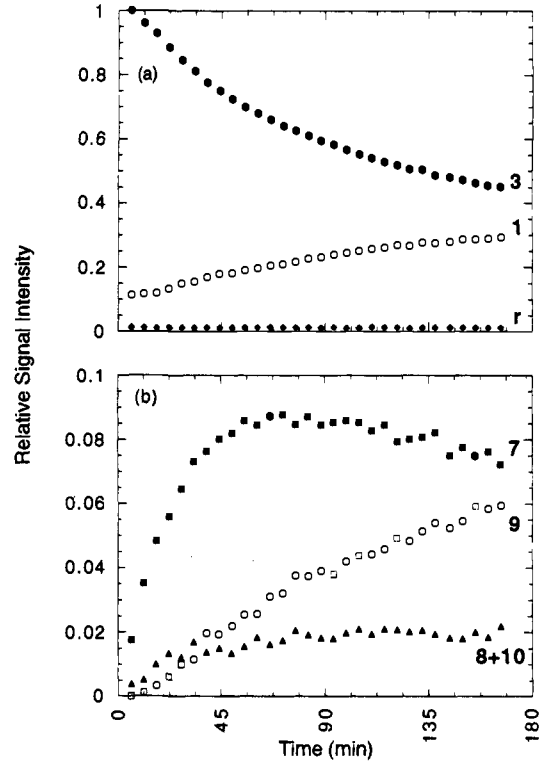
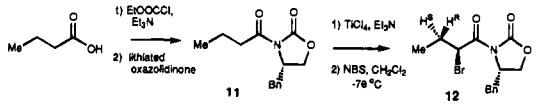


Figure 5. Relative $^{1}H-^{13}C$ cross peak signal intensities of substrate and products derived from (2*S*)-[3-^{13}C]isobutyryl-CoA (**3**) as monitored by 2D NMR (see Figure-3). The signal intensities are relative to that of the substrate (**3**) at the first measurement (5.75 min). Note the 10-fold decrease of the ordinate scale in (b) relative to that in (a). The signal assignments are those referred to in Figure-4.

It should be noted, that these time courses represent integrated signal intensities from the 2D $^{1}H-^{13}C$ shift correlation spectra, which can be directly related to the relative molar ratios of the products formed. These spectra also indicate that the relative levels of ICM and thioesterase activity in this enzyme extract (prepared by ammonium sulfate fractionation, and four subse-

Scheme 2



quent chromatographic steps) are on about the same order of magnitude.

Turnover of [2-^{2}H]isobutyryl-CoA. To follow the stereochemical course of the ICM reaction at C2 in isobutyryl-CoA, [2-^{2}H]isobutyryl-CoA (98% ^{2}H enrichment) was incubated over ≈3 h with the enzyme extract. The isobutyrate recovered from the incubation after thioester hydrolysis retained only ≈75% deuterium enrichment at C2, thereby showing partial exchange of ^{2}H with ^{1}H from the solvent. This exchange reaction (but not the rearrangement) occurred in the absence of coenzyme B_{12}, but not in the absence of enzyme extract, showing that the exchange *is not catalyzed by a coenzyme B_{12}-dependent enzyme.* The incubation of (2*S*)-[3-^{13}C]isobutyryl-CoA in the enzyme extract without added coenzyme B_{12}, described above, showed that the exchange at C2 occurs stereospecifically, *i.e., the enantiomeric purity of the ^{13}C-labeled substrate is not altered, within the limits of the NMR asssay, by the exchange process.*

The *n*-butyryl-CoA derived from [2-^{2}H]isobutyryl-CoA should be deuterium labeled at both of the stereoheterotopic positions at C3. After thioester hydrolysis, this [3-^{2}H$_1$]*n*-butyrate was recovered, diluted with unlabeled *n*-butyrate, and converted into the corresponding deuterated form of N-butyryloxazolidone **11** (Scheme 2). Bromination of the titanium enolate derived from **11** gave stereoselectively the stable (2*S*)-bromide **12**.[14,15] The diastereotopic protons at C3 in **12** resonate at 2.05 and 2.21 ppm in the ^{1}H NMR spectrum (in DMSO-d_6). To allow a stereospecific assignment of these signals, two stereospecifically ^{2}H$_1$ labeled derivatives were prepared in the same way, starting from (3*R*)- and (3*S*)-[3-^{2}H$_1$]*n*-butyrate, which in turn were prepared by the method of Bücklers et al.;[16] the 3-pro-*R* and 3-pro-*S* protons in **12** could then be assigned to resonances at 2.05 and 2.21 ppm, respectively. The ^{2}H NMR spectrum of [3-^{2}H$_1$]-**12** derived from the enzymically produced [3-^{2}H$_1$]*n*-butyrate showed deuterium in the 3-pro-*R* and 3-pro-*S* positions in a 3:2 ratio.

Discussion

The coenzyme B_{12}-dependent mutases comprise a small group of enzymes that promote rearrangements in which a group (-X) migrates to an adjacent carbon, while a hydrogen atom undergoes a 1,2-migration in the opposite direction, thereby leading to a vicinal interchange of -H and -X. Three mutases (apart from ICM) catalyze carbon skeleton rearrangements in this way and have shown localized regions of similarity in their primary sequences, namely, MCM,[6] glutamate mutase,[17-19] and 2-methyleneglutarate mutase.[20] The ICM reaction, on the other hand, is clearly related to that of MCM (Figure 1), and it seems likely that these two enzymes will prove to be closely related at the structural and genetic levels. Efforts are presently underway in our laboratory to clone and sequence the ICM gene, based upon primary sequence data derived from tryptic fragments of the purified enzyme from *S. cinnamonensis*.[7]

Stereochemical studies have shown that the predominant course of the glutamate mutase[21,22] and 2-methyleneglutarate mutase[23] reactions at C2 is inversion, whereas that of MCM[9] is retention. However, studies with isotopically labeled methylmalonyl-CoA and methylmalonylcarba(dethia)-CoA indicate either that the ability of MCM to distinguish between the 3-pro-*R* and 3-pro-*S* hydrogens in succinyl-CoA during its conversion into (2*R*)-methylmalonyl-CoA is not strict,[24] or that intervening 1,2-H shifts with inversion at the receiving site may occur in substrates bound at the active site of the enzyme.[25] Moreover, stereochemical studies on other coenzyme B_{12}-dependent enzymes, including ethanolamine ammonia lyase[26,27] and diol dehydrase,[28] have provided evidence for the occurrence of alternative binding modes for substrates and bound radical intermediates that may result in a racemization of labeled substrates during turnover on these enzymes.

With this background, and the observation from whole-cell feeding experiments that the ICM reaction may not occur stereospecifically (see the Introduction), it was of interest to investigate the stereochemistry of this reaction *in vitro*. A partially purified ICM has been used for this work. This was necessary, since homogeneous ICM is available only in very poor yield from *S. cinnamonensis* (data not presented), yet large amounts of labeled isobutyryl-CoA must be turned over, to allow further chemistry and NMR analyses to be performed on the *n*- and isobutyrate portions of the products (see above). Although care must be exercised in using only a partially purified enzyme, a strict dependence of the rearrangement and of the ensuing loss of stereospecificity on added coenzyme B_{12} was observed, i.e., a coenzyme B_{12}-dependent enzyme is responsible for both.

The results described above show that during the ICM reaction the carbonylthioester moiety of isobutyryl-CoA migrates preferentially to the pro-*S* methyl of the isobutyryl group, consistent with earlier whole-cell feeding experiments.[8] However, a migration of the carbonylthioester must also occur, albeit at a lower rate, to the pro-*R* methyl group of isobutyryl-CoA, since (2*S*)-[3-^{13}C]isobutyryl-CoA is converted into [4-^{13}C] as well as into [2-^{13}C]*n*-butyryl-CoA. With prolonged reaction times, the proportion of [4-^{13}C]*n*-butyryl-CoA increases as equilibrium is approached. At present, no convenient spectrophotometric assay is available for accurate kinetic measurements of ICM activity. However, a series of 2D inverse detected ^{1}H-^{13}C correlation spectra measured during the turnover of (2*S*)-[3-^{13}C]isobutyryl-CoA (Figures 3 and 4) allowed the simultaneous detection of both rearrangement products (i.e., **7** and **8**) as well as the products of thioester hydrolysis (i.e., **1**, **9**, and **10**). These data show that the formation of **7** is significantly faster than the appearance of **8** (Figure 5), the difference being most likely at least an order of magnitude.

A similar picture emerges when the stereochemical course of the reaction is monitored at C2 of isobutyryl-CoA. Here,

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the turnover of [2-^{2}H]isobutyryl-CoA was allowed to proceed for several hours, to provide sufficient [3-$^2H_1$]*n*-butyryl-CoA for analysis. After conversion of this [3-$^2H_1$]*n*-butyrate into the corresponding deuterated form of oxazolidinone **12** (Scheme 2), ^{2}H NMR spectroscopy showed that 60% of the product had the (3*R*)-[3-$^2H_1$] and 40% the (3*S*)-[3-$^2H_1$] absolute configuration. Thus, the major product of the coenzyme B_{12}-dependent ICM arises by migration of the carbonylthioester group in [2-^{2}H]-isobutyryl-CoA to the pro-*S* methyl group, which is replaced by a hydrogen atom with retention. However, a significant amount of product also arises formally by inversion. The proportion of this inversion product is expected to increase as equilibrium is approached, due to a kinetic isotope effect providing isotopic discrimination in the reverse reaction; (3*R*)-[3-$^2H_1$]*n*-butyryl-CoA should be converted back to [2-^{2}H]-isobutyryl-CoA more rapidly than the (3*S*) isomer, which accumulates.

An enzyme catalyzed loss of deuterium through protium exchange at the C2 position of isobutyryl-CoA was also detected. However, this exchange occurs stereospecifically and in the absence of coenzyme B_{12}; it was shown also that (2*S*)-[3-^{13}C]isobutyryl-CoA under the same incubation conditions does not suffer loss of stereochemical purity—this is an important result in the light of the observations (vide supra) made during the transformation of (2*S*)- and (2*R*)-[3-^{13}C]-isobutyryl-CoA. Possibly this exchange is catalyzed by trace amounts of isobutyryl-CoA dehydrogenase, which is produced by the microorganism to catabolize valine,[29] a major constituent of the growth medium. Attempts to detect this dehydrogenase in the enzyme extract used for the turnover experiments (using a spectrophotometric assay with PMS and DCIP as intermediate and terminal electron acceptors[30]) were unsuccessful. Nevertheless, the presence of trace amounts of the dehydrogenase below the levels detectable by this assay cannot be ruled out. Related acyl-CoA dehydrogenases are known to promote a stereospecific proton-deuteron exchange at C2 in the absence of enoyl-CoA product formation.[31] So although the catalyst responsible for the exchange is not proven, an isobutyryl-CoA dehydrogenase (at low levels) remains the most likely culprit.

The partial scambling of both ^{13}C and ^{2}H label observed in these experiments can be reconciled with the generally accepted mechanism of the MCM reaction. We proceed from the assumption that the MCM and ICM reactions will have similar, if not identical, mechanisms, which involve, firstly, homolysis of the Co−C bond in coenzyme B_{12} and abstraction of a hydrogen atom from a methyl group in the substrate (i.e., methylmalonyl-CoA or isobutyryl-CoA) by the 5′-deoxyadenosyl radical (Figure 6). The generation of Co(II) and organic free radicals initiated by substrate binding to MCM has been established by ESR measurements, using both methylmalonyl-CoA[32,33] and several substrate analogues including succinylcarba(dethia)-CoA.[34,35] With ICM, the substrate isobutyryl-CoA may bind at the active site such that the pro-*S* methyl group is

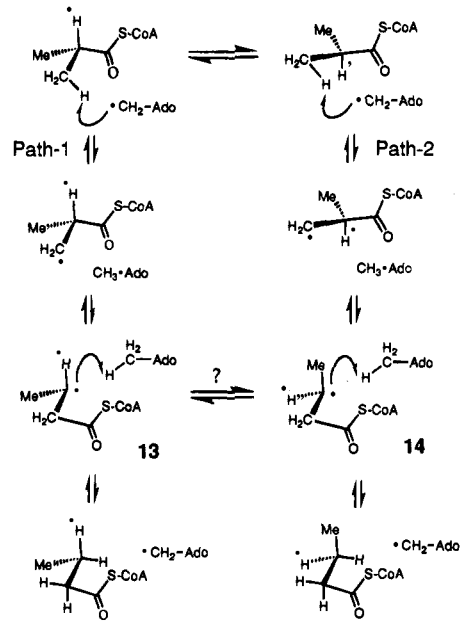

Figure 6. Possible stereochemical paths for the turnover of isobutyryl-CoA on ICM (see text).

preferentially attacked by the 5′-deoxyadenosyl radical. However, a slower hydrogen atom abstraction should also occur from the pro-*R* methyl group, most likely in an alternative conformation (Figure 6). In the MCM reaction, the presence of a carboxylate group at C3 in methylmalonyl-CoA should facilitate the positioning of substrate in the active site, consistent with a strict stereospecificity of MCM toward the (2*R*) epimer of methylmalonyl-CoA.[24]

After hydrogen atom abstraction from a methyl group in isobutyryl-CoA, the carbonylthioester group should migrate to generate a product methylene radical at C3 of the new *n*-butyryl moiety. It may be no coincidence that the extent of scrambling of ^{13}C label, observed during turnover of [3-^{13}C]isobutyryl-CoA, is approximately the same as that of ^{2}H label during transformation of [2-^{2}H]isobutyryl-CoA. This could occur if the substrate, isobutyryl-CoA, is turned over at the active site of ICM in two alternative conformations, from each of which a stereospecific 1,2-migration of the COSCoA and back migration of a hydrogen atom from 5′-methyladenosine occur, i.e., each with retention with no crossover between paths 1 and 2 shown in Figure 6. However, it is also conceivable that the product methylene radical may interconvert between two conformational states (i.e., **13** ↔ **14**) prior to H-atom transfer from 5′-methyladenosine. Alternatively, the observed loss of stereocontrol in the turnover of [2-^{2}H]isobutyryl-CoA could also arise due to an intervening 1,2-H shift with inversion at the receiving site, and donation of a hydrogen atom from 5′-methyladenosine to C2, analogous to that postulated to occur during the turnover of methylmalonyl-carba(dethia)-CoA *and* propionylcarba(dethia)-CoA on MCM.[25] Whatever the correct interpretation is in this case, the observed loss of stereocontrol in the conversion of these labeled isobutyryl-CoAs finds precedence in related observations of substrate turnover on MCM. More detailed studies may be facilitated in the future by the availability of recombinant ICM, and experiments to realize this goal are in progress.

Finally, the results described above illustrate the advantages of gradient-enhanced, inverse detected heteronuclear 2D ^{1}H−^{13}C correlation spectroscopy in following the ICM and thioesterase reactions simultaneously in the NMR tube. This NMR experi-

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ment has a high intrinsic sensitivity when ^{13}C labeled substrate is used and extended phase cycling is obviated, so the acquisition of each 2D spectrum takes only a few minutes measuring time. Also, each 2D spectrum has much improved spectral resolution compared to its two 1D analogues, aiding in the assignment and integration of signals. The use of field gradients also aids significantly in suppressing unwanted signals from protons not bonded to ^{13}C, which minimizes t_1 noise and allows use of protium-containing additives such as glycerol and β-mercaptoethanol, in H_2O, to stabilize the enzyme. Given the quality and quantity of data obtained, the extraction of accurate kinetic constants for the ICM reaction using this technique may also be feasible. Clearly, more complicated metabolic networks could be qualitatively and quantitatively investigated in this way.

Experimental Section

General Methods and Equipment. ^1H, ^2H, and ^{13}C NMR spectra were recorded on Bruker AC300 (^1H 300 MHz) or AMX600 (^1H 600 MHz) spectrometers at 300 or 303 K. Chemical shifts in ppm using residual solvent signal as internal standard (^1H, ^2H: 7.24 for $CDCl_3$; 2.50 for DMSO; 4.80 for H_2O/D_2O. ^{13}C: 77.0 for $CDCl_3$) are given on the δ scale. Ultraviolet (UV) measurements were made with a Varian Cary-3 spectrophotometer. Mass spectra were determined on a Varian MAT-112S (CI with NH_3) or Finnigen TSQ 700 (electrospray). Gas chromatography (GC) used a Hewlett Packard HP-5890 series II chromatograph with an HP-FFAP column (10 m × 0.53 mm). GC-MS used the same GC system with a HP-5 column (25 m × 0.2 mm) coupled to a Hewlett Packard 5971 mass selective detector. HPLC was carried out on a Pharmacia system with a Waters RCM-μBondapak C18 cartridge (25 × 100 mm). All operations with coenzyme B_{12} were carried out in the dark or under dim light. *Streptomyces cinnamonensis* A3823.5 was obtained from Eli Lilly. Diethyl ether and THF were purified by distillation from Na/benzophenone, and chloroform was distilled from CaH_2 before use.

General procedure for the Synthesis of *n*- and Isobutyryl-CoA. Sodium butyrate (15 mg, 136 μmol) and *p*-toluenesulfonic acid (29 mg, 150 μmol) in dry THF (2 mL) was stirred at room temperature for 2 h. *N,N'*-Carbonyldiimidazole (25 mg, 154 μmol) was added with stirring overnight. After evaporation of the THF *in vacuo*, the resulting powder was redissolved in aqueous KH_2PO_4 (1 mL, 100 mM, pH 7.4). Coenzyme A (40 mg, 46.2 μmol) was added, and the solution stirred for 4 h at room temperature. The product was purified by reverse-phase HPLC with a gradient of 5−40% MeOH in 25 mM KH_2PO_4, pH 5.5 over 30 min (flow rate 8 mL/min, UV detection at 254 nm, t_r 20−25 min). To desalt the product, HPLC using a gradient of 5−40% MeOH with pure water (t_r 10−12 min) was performed. *n*-Butyryl-CoA: ϵ = 16 800 (259.5 nm); ^1H NMR (10% D_2O in H_2O, pH 5.5, 600 MHz) δ 0.69 (s, 3H), 0.80 (t, J = 7.3, 3H), 0.81 (s, 3H), 1.52 (m, 2H), 2.36 (t, J = 6.6, 2H), 2.48 (t, J = 7.3, 2H), 2.90 (t, J = 6.3, 2H), 3.25 (m, 2H), 3.38 (m, 2H), 3.49 (dd, J = 9.9, 5.0, 1H), 3.75 (dd, J = 9.9, 5.0, 1H), 3.94 (s, 1H), 4.17 (m, 2H), 4.52 (m, 1H), 4.75 (m, 2H), 6.10 (d, J = 6.4, 1H), 7.92 (m, 1H), 8.06 (m, 1H), 8.20 (s, 1H), 8.47 (s, 1H). Isobutyryl-CoA: ^1H NMR (10% D_2O in H_2O, pH 5.5, 600 MHz) δ 0.65 (s, 3H), 0.78 (s, 3H), 1.02 (d, J = 7.4, 6H), 2.32 (t, J = 6.6, 2H), 2.67 (septet, J = 7.4, 1H), 2.87 (t, J = 6.3, 2H), 3.21 (t, J = 6.3, 2H), 3.35 (m, 2H), 3.45 (dd, J = 9.9, 5.0, 1H), 3.72 (dd, J = 9.9, 5.0. 1H). 3.90 (s, 1H), 4.15 (m, 2H), 4.49 (m, 1H), 4.70 (m, 2H), 6.07 (d, J = 6.4, 1H), 8.14 (s, 1H), 8.46 (s, 1H). (2*S*)-[3-^{13}C]Isobutyryl-CoA (**3**): ^1H NMR (10% D_2O in H_2O, pH 5.5) δ 0.99 (dd, J = 7, 5, 3H), 1.00 (dd, J = 128, 7.0, 3H), 2.66 (m, 1H), otherwise as above. (2*R*)-[3-^{13}C]Isobutyryl-CoA (**4**): ^1H NMR (10% D_2O in H_2O, pH 5.5) δ 0.99 (dd, J = 7.0, 5.1, 3H), 1.00 (dd, J = 128, 7.0, 3H). 2.66 (m, 1H), otherwise as above.

Enzyme Assays. ICM was assayed as previously described.[5] Thioesterase activity was detected according to the Ellman assay for free thiols.[36]

Preparation of ICM. *S. cinnamonensis* A3823.5 was fermented in 15 l batches in a Chemap CF3000 fermentor, as previously described.[39] The wet cell paste (≈700 g) could be stored at −80 °C. Disruption of the cells (per 100 g wet wt) in buffer (150 mL, 100 mM KH_2PO_4, 10 mM EDTA, 25% glycerol, 1 mM DTT, 1 mM PMSF, 0.1% β-mercaptoethanol, 1 mM benzamidine, pH 7.4) was accomplished by sonication at ≤10 °C (Heat System model W-220F ultrasonic cell disrupter). The extract was centrifuged (25 000 × g, Beckman GSA rotor, 60 min) to remove cell debris. Ammonium sulfate was added with stirring at 4 °C over 1 h to 35% saturation, during which time the pH was maintained at 7.4 (10 M KOH). The solution was stirred overnight at 4 °C and then clarified by centrifugation. The supernatant was taken to 70% ammonium sulfate saturation in the same manner and the resulting pellet was collected and stored at −80 °C. The protein pellet (from ≈250 g cells) was dissolved in 50 mL buffer A (50 mM KH_2PO_4, 5 mM EDTA, 5% glycerol, 1 mM DTT, 0.05% β-mercaptoethanol, pH 7.4) and applied at 3 mL/min to a DEAE Sepharose column (Pharmacia FastFlow, 2.6 × 45 cm) at 4 °C. After a 75 mL wash with buffer A, a 0-1 M KCl gradient was applied over 500 mL followed by a 150 mL wash with 1 M KCl in buffer A. Fractions (10 mL) were collected with UV-detection at 280 nm. Pooled fractions were concentrated to 2−3 mL (Amicon Centriprep 10 concentrators, Sorvall TechnoSpin R centrifuge, 4000 rpm, 4 °C) and dialyzed overnight against buffer A at 4 °C before assaying. Fractions containing IBM activity were pooled and further concentrated to a total volume of 10−15 mL.

ICM Turnover of (2*R*)- and (2*S*)-[3-^{13}C]Isobutyryl-CoA. Coenzyme B_{12} (380 μL, 5 μM) and then **4** (32 mg, 38.2 μmol) in H_2O (2.5 mL) were added to partially purified ICM (200 mg protein in 25 mL buffer A) in the dark. After shaking for 3 h at 30 °C, the solution was adjusted to pH 10 with 4 M NaOH (0.5 mL) and stirred for 10 min. 25% H_2SO_4 (0.5 mL) was then added, and the solution extracted with ether (3 × 10 mL). The ether extract was assayed by GC to determine the extent of conversion of iso- to *n*-butyrate (iso:*n* = 3:1). The ether was removed in vacuo, and the butyric acids were dissolved in acetone (2 mL). *p*-Bromophenacylbromide (15 mg, 54 μmol) and Et_3N (15 μL, 108 μmol) were then added, and the solution was stirred overnight at room temperature. The resulting *p*-bromophenacyl esters were purified by preparative TLC (silica gel-60, cyclohexane:$CHCl_3$:Et_2O 6:1:2). *p*-Bromophenacyl-[^{13}C]*n*-butyrate: ^1H−decoupled inverse gated ^{13}C NMR ($CDCl_3$, 150 MHz) δ 13.7 (67C, C4), 18.4 (1C, d, J = 35, C3), 35.8 (33C, C2), 65.6 (1C, C1'), 129.1, 129.2, 132.2, 133.0, 173.0, 191.4. The *p*-bromophenacyl [3-^{13}C]isobutyrate recovered from the incubation of **4** was then stirred in MeOH (0.5 mL) with 2 N NaOH (0.1 mL) for 1 h at room temperature. Water was then added, and the solution was washed with EtOAc, acidified with HCl, and extracted with EtOAc. The EtOAc extract was dried ($MgSO_4$) and concentrated. The [3-^{13}C]isobutyric acid was then esterified with (*S*)-(+)-methyl mandelate and purified, as described previously:[8] ^1H-decoupled inverse gated ^{13}C NMR ($CDCl_3$, 150 MHz) δ 18.6 (17C, C3-pro-*S*), 18.8 (83C, C3-pro-*R*). Similarly, **3** (34 mg) was converted by ICM in the presence of coenzyme B_{12} into [^{13}C]butyric acid (33% conversion of iso- to *n*-butyrate by GC assay), and then into the *p*-Bromophenacyl ester, which was purified by TLC. *p*-bromophenacyl-[^{13}C]*n*-butyrate: ^1H-decoupled inverse gated ^{13}C NMR ($CDCl_3$, 150 MHz) δ 13.7 (26C, C4), 18.4 (1C, d, J = 35, C3), 35.8 (74C, C2), 65.6 (1C, C1'), 129.1, 129.2, 132.2, 133.0, 173.0, 191.4.

ICM turnover of [2-^2H]isobutyryl-CoA. As above, [2-^2H]isobutyryl-CoA (58 mg) was converted by ICM into [3-$^2H_1$]butyryl-CoA. After hydrolysis of the thioesters at pH 10, and extraction of the acids into EtOAc, GC revealed a 3:1 ratio of iso- to *n*-butyric acids. Unlabeled *n*-butyric acid (10 mg) was added to the mixture of deuterated butyric acids, and the sodium salts were generated by extracting the acids into 1 N NaOH. The solution was lyophilized, and the sodium butyrates were converted into the oxazolidinones, as described below for **11**, to give a 7:3 mixture of the *n*-:isobutyryl oxazolidinone derivatives (12.5 mg). The [2-^2H]isobutyryl oxazolidinone showed by GC-MS *m/z* 248 (M^+, 100), 247 (29). The (3(2*S*),4*S*)-3-([3-$^2H_1$]-2-bromo-1-oxobutyl)-4-(phenylmethyl)-2-oxazolidinone was then prepared as described for **12** and purified by reverse-phase HPLC (70% MeOH, 6 mL/min, 254 nm): ^2H NMR (DMSO, 92 MHz) δ 2.05 (1.5D), 2.21 (1.0D). In a separate experiment, [2-^2H]isobutyryl-CoA (2 mg)

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was partially converted by the mutase into [3-$^2H_1$]*n*-butyryl-CoA. After hydrolysis of the thioesters, the acids were extracted and converted directly into their *p*-bromophenacyl esters, as described above. *p*-Bromophenacyl [2-^{2}H]isobutyrate: GC-MS *m/z* 287 (M^+, 100), 286 (47), 285 (M^+, 90), 284 (27). *p*-Bromophenacyl [3-^{2}H]*n*-butyrate: GC-MS *m/z* 287 (M^+, 100), 286 (54), 285 (M^+, 77), 284 (38).

(4*S*)-3-(1-Oxobutyl)-4-(phenylmethyl)-2-oxazolidinone (11). To sodium *n*-butyrate (100 mg, 0.90 mmol) and *p*-toluenesulfonic acid (189 mg, 0.99 mmol) in dry THF (5 mL), was added triethylamine (250 μL, 1.80 mmol) and ethylchloroformate (130 μL, 1.35 mmol) at 0 °C and stirred for 1 h. Meanwhile, (*S*)-4-benzyl-2-oxazolidinone (239 mg, 1.35 mmol) and triphenylmethane (10 mg) were dissolved in THF (2 mL) and cooled to −78 °C. *n*-Butyllithium (1.5 M in *n*-hexane, 900 μL, 1.35 mmol) was slowly added to the solution over 10 min until a red color persisted. The solution containing mixed anhydride was then cooled to −78 °C, and the lithiated oxazolidinone was added via canula. After stirring overnight at room temperature, the mixture was poured into ether, washed sequentially with 10% citric acid in brine and $NaHCO_3$ in brine and brine, dried (Na_2SO_4), and concentrated to give a yellow oil. Purification by silica flash chromatography (hexane:EtOAc 4:1) gave the product as a clear, colorless oil (151 mg, 68% yield): ^{1}H NMR ($CDCl_3$) δ 1.02 (t, *J* = 6.8, 3H), 1.73 (q, *J* = 6.8, 2H), 2.77 (dd, *J* = 13.3, 9.5, 1H), 2.91 (m, 2H), 3.26 (dd, *J* = 13.2, 3.3, 1H), 4.14−4.24 (m, 2H), 4.71 (m, 1H), 7.22−7.36 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 13.6, 17.7, 37.3, 37.9, 55.1, 66.1, 126.2, 127.3, 128.2, 128.9, 129.4, 135.3, 153.0, 173.2; CI MS *m/z* 248 ($M^+ + 1$). Anal. Calcd for $C_{14}H_{17}NO_3$: C, 68.02; H, 6.88; N, 5.67. Found: C, 67.79; H, 6.80; N, 5.65.

(3(2*S*),4*S*)-3-(2-Bromo-1-oxobutyl)-4-(phenylmethyl)-2-oxazolidinone (12). $TiCl_4$ (4.9 μL, 44.6 μmol) was added to a solution of **11** (10.5 mg, 42.5 μmol) in dry CH_2Cl_2 (1 mL) at 0 °C. After stirring for 20 min, Et_3N (7.1 μL, 51.0 μmol) was added and the solution was stirred for 1 h. After cooling to −78 °C, the dark red solution immediately turned light yellow upon addition of NBS (8.4 mg, 46.8 μmol) in CH_2Cl_2 (1 mL). After 15 min, the reaction was quenched with the addition of 5% sodium thiosulfate (5 mL) and ether (10 mL). After extraction once more with 5% sodium thiosulfate and then water (2 × 5 mL) and drying ($MgSO_4$), the solvent was removed *in vacuo*. Silica flash chromatography (hexane:EtOAc 6:1) gave the product (6.2 mg): ^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.05 (t, *J* = 7.2, 3H, H4), 2.08 (m, 1H, H3-pro*R*), 2.16 (m, 1H, H3-pro*S*), 2.79 (dd, *J* = 13.2, 9.6, 1H), 3.31 (dd, *J* = 13.2, 2.2, 1H), 4.20 (dd, *J* = 9.0, 3.2, 1H), 4.22 (t, *J* = 9.0, 1H), 4.71 (tt, *J* = 8.8, 4.6, 1H), 5.54 (t, *J* = 7.0, 1H), 7.20−7.35 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 11.9, 27.3, 37.0, 45.7, 55.2, 66.2, 127.4, 129.0, 129.5, 134.8, 152.2, 169.1; MS *m/z* 327 (M^+, 36), 325 (M^+, 37), 151 (97), 149 (100).

Sodium (3*S*)-[3-$^2H_1$]Butyrate. Lithium aluminium deuteride (1.5 g, 35 mmol) in ether (50 mL) was cooled to −10 °C. An ice cold solution of ethyl (3*R*)-3-brosylbutyrate[16] (8.7 g, 26 mmol) in ether (10 mL) was then added. After warming to room temperature overnight, the excess $LiAlD_4$ was quenched with saturated aqueous $MgSO_4$ (3 mL). After filtration through Celite and drying (Na_2SO_4), the ether was removed by short path distillation. Sulfuric acid (5 mL, concentration) was added to chromium oxide (5.3 g, 53 mmol) in water (15 mL) at 0 °C. The deuterated butanol in water (15 mL) was then added slowly, keeping the temperature near 0 °C. The solution was allowed to warm to room temperature, and then the volatile components were removed *in vacuo*. The solution was then adjusted to pH 9 with 1 M NaOH and lyophilized to give the product as a white powder (1.58 g), contaminated with sodium acetate: ^{1}H NMR (D_2O) δ 0.85 (d, *J* = 7.4, 3H), 1.49 (m, 1H), 1.88 (s, acetate), 2.11 (d, *J* = 7.3, 2H); ^{13}C NMR (D_2O) δ 13.5, 19.3 (t, *J* = 19.7), 23.6 (acetate), 39.9, 181.9, 184.4 (acetate).

Sodium (3*R*)-[3-$^2H_1$]Butyrate. Prepared from ethyl (*S*)-3-brosylbutyrate by the procedure described above: ^{1}H NMR (D_2O) δ 0.85 (d, *J* = 7.4, 3H), 1.49 (m, 1H), 1.88 (s, acetate), 2.11 (d, *J* = 7.3, 2H); ^{13}C NMR (D_2O) δ 13.5, 19.3 (t, *J* = 19.6), 23.6 (acetate), 39.9, 181.9, 184.4 (acetate).

(3(2*S*,3*S*),4*S*)-3-([3-$^2H_1$]-2-Bromo-1-oxobutyl)-4-(phenylmethyl)-2-oxazolidinone. Prepared from **sodium (3*S*)-[3-$^2H_1$]butyrate** as described above for **12**: ^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.05 (d, *J* = 7.5, 3H), 2.06 (pentet, *J* = 7.4, 1H), 2.79 (dd, *J* = 13.2, 9.6, 1H), 3.31 (dd, *J* = 13.2, 2.2, 1H), 4.20 (dd, *J* = 9.0, 3.2, 1H), 4.22 (t, *J* = 9.0, 1H), 4.71 (tt, *J* = 8.8, 4.6, 1H), 5.53 (d, *J* = 7.8, 1H), 7.20−7.35 (m, 5H); ^{2}H NMR (DMSO, 92 MHz) δ 2.21.

(3(2*S*,3*R*),4*S*)-3-([3-$^2H_1$]-2-Bromo-1-oxobutyl)-4-(phenylmethyl)-2-oxazolidinone. Prepared from **sodium (3*R*)-[3-$^2H_1$]butyrate** as described above for **12**: ^{1}H NMR ($CDCl_3$, 600 MHz) δ 1.05 (d, *J* = 7.5, 3H), 2.14 (m, 1H), 2.79 (dd, *J* = 13.2, 9.6, 1H), 3.31 (dd, *J* = 13.2, 2.2, 1H), 4.20 (dd, *J* = 9.0, 3.2, 1H), 4.22 (t, *J* = 9.0, 1H), 4.71 (tt, *J* = 8.8, 4.6, 1H), 5.53 (d, *J* = 6.3, 1H), 7.20−7.35 (m, 5H); ^{2}H NMR (DMSO, 92 MHz) δ 2.05.

2D-NMR. Substrate (**3**) (11 μmol) in D_2O (50 μL) was added to coenzyme B_{12} (0.25 μmol) and partially purified ICM (19 mg protein) in buffer A (450 μL) just prior to the acquisition of the first 2D spectrum. The enzyme was partially purified by the method described above and then further by ion exchange (Q-Sepharose) and gel filtration (Superdex-200 and TSK-G3000SWG) chromatography. Spectra were acquired on a Bruker AMX600 instrument equipped with a triple resonance gradient probehead and Z-gradient hardware at 30 °C. Data acquisition started 3 min after insertion of the sample into the magnet. A version of the pulse sequence of Vuister & Bax[13] was used, with sine-shaped gradient pulses of 1.5 ms duration. GARP decoupling[37] was applied during t_2. Spectral widths were 4505 Hz in the ^{1}H dimension and 7530 Hz in the ^{13}C dimension. Complex point (1024) in t_2 and 64 complex t_1 points were acquired using the TPPI-States scheme[38] for quadrature detection in the indirect dimension. Two scans were averaged per t_1 point, giving a total duration of 5.5 min for each 2D spectrum. Thirty spectra were acquired in immediate succession, thus monitoring the reaction over nearly 3 h.

Acknowledgment. This work was supported by grants from the Swiss National Science Foundation and from the Royal Society (London) (to A.B.). We are particularly grateful to K. Burkhardt for assistance with the enzyme purification and Professor Janos Rétey for critical reading of the manuscript.

Supporting Information Available: Experimental details for the synthesis of ^{13}C labeled isobutyrates shown in Scheme 1 (3 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

JA952394D