6-Methyl-1,2,3,4-tetrahydroazulen-1-ol (**8c**), obtained from trienone **7c** by AlH_3 reduction as above (92%), was treated similarly (temperature = 445 °C, nitrogen flow rate = 3.3 L/min, addition rate = 1 mL/8 s) to give 6-methylazulene (**9c**)[25] in 22% yield.

1,6-Dimethyl-1,2,3,4-tetrahydroazulen-1-ol (**8d**), obtained from trienone **7d** by CH_3MgCl addition as above (96%), was treated similarly (temperature = 428 °C, nitrogen flow rate = 2.2 L/min, addition rate = 1 mL/18 s) to give 1,6-dimethylazulene (**9d**) in 15% yield. A pure sample of this previously unknown azulene derivative was obtained as a blue oil by preparative GLC. Anal. Calcd for $C_{12}H_{12}$: C, 92.26; H, 7.74. Found: C, 91.99; H, 7.91. UV-Vis max (hexane): 270 nm (ϵ 60 000), 276 (61 000), 301 (7100), 349 (5100), 366 (2200), 550 (sh, 230), 573 (270), 595 (320), 622 (280), 651 (290), 688 (130), 722 (130) [Vis max predicted by Plattner's empirical rules:[26] calcd, 594 nm; found, 595 nm].

(25) Identified by comparison of the rich spectrum with published data: Plattner, P. A.; Heilbronner, E. *Helv. Chim. Acta* **1947**, *30*, 910-20.

Acknowledgment. We wish to thank the National Science Foundation, the University of Nevada Research Advisory Board, the National Institutes of Health (Grant NCI-CA-23488), and the donors of the Petroleum Research Fund, administered by the American Chemical Society, for financial support of this work.

(26) Plattner, P. A. *Helv. Chim. Acta* **1941**, *24*, 283E-94E.

Stereochemistry of Indolmycin Biosynthesis. Steric Course of C- and N-Methylation Reactions[1a]

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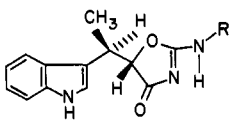
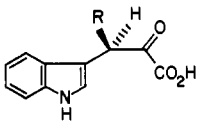
Abstract: (2*S*,*methyl-R*)- and (2*S*,*methyl-S*)-[*methyl*-^{14}C,$^{2}H_1$,$^{3}H_1$]methionine were synthesized from (*S*)- and (*R*)-[2-^{14}C,$^{2}H_1$,$^{3}H_1$]acetate. Key steps in the synthesis were Schmidt reaction of the acetate to give methylamine and use of methylditosylimide, derived from the latter, to alkylate the *S* anion of L-homocysteine. The two chiral methionine samples were fed to cultures of *Streptomyces griseus* producing the antibiotic indolmycin (Ia) and its precursor indolmycenic acid (III). Both compounds were degraded to convert the *C*- and the *N*-methyl groups into the methyl group of acetic acid, using reactions or reaction sequences of known stereochemistry. Chirality analysis of these acetic acid samples by the method of Cornforth et al. and Arigoni and co-workers indicated that the enzymatic transfer of the methyl group of (*S*)-adenosylmethionine both to carbon and to nitrogen as acceptor occurs with inversion of configuration.

The biosynthesis of the antibiotic indolmycin (Ia) by *Streptomyces griseus* (ATCC 12648) has been shown[2,3] to involve the transfer of the methyl group of methionine, in the form of (*S*)-adenosylmethionine, to the methylene carbon of indolpyruvate (IIa), which is generated by transamination of tryptophan, and



Ia: R = CH_3; indolmycin
b: R = H; N-desmethylindolmycin



IIa: R = H; indolepyruvate
b: R = CH_3; 3-methylindolepyruvate

to the exocyclic nitrogen of the amidino group in the oxazolinone ring of *N*-demethylindolmycin (Ib). The biosynthesis of indolmycin poses several stereochemical questions. The answers to some of these are evident, but several others involve cryptic stereochemistry, because they relate to reactions at centers which are not chiral. The latter reactions nevertheless can be expected to be stereospecific and their steric course can be deduced by experiments using suitable stereospecifically isotope-labeled precursors. The main stereochemical questions are (a) does replacement of a methylene hydrogen in IIa by a methyl group proceed in an inversion or retention mode at C-3, (b) does the methyl group itself undergo inversion or retention of configuration (or racemization) upon transfer to the methyl carbon, and (c) does the methyl group transfer to the nitrogen occur with inversion, retention or racemization. Question a was answered in previous work from this laboratory.[4] In this paper we report results which answer questions b and c. A preliminary account of some of this work has been published earlier.[5]

Results

The experimental approach to the study of the stereochemical fate of the methyl group of methionine in indolmycin biosynthesis required use of the chiral methyl group methodology[6] developed by the laboratories of Cornforth[7] and Arigoni.[8] This method involves the use of all three isotopes of hydrogen (^{1}H, ^{2}H, ^{3}H) to generate a methyl group which is chiral by virtue of isotopic substitution. For the present study the following three tasks had to be performed: (a) synthesis of methionine carrying a chiral methyl group of known absolute configuration, (b) incorporation of the chiral methyl group of methionine into Ia, and (c) degradation of Ia to convert each methyl group into the methyl group of acetate by a series of stereochemically unambiguous reactions followed by chirality analysis of these acetate samples. The latter was carried out as indicated by the laboratories of Cornforth[7] and

(1) (a) This work was supported by the U.S. Public Health Service through Research Grant GM 18852 and postdoctoral fellowship GM 06695 (to R. W.W.) from the National Institutes of Health.
(2) U. Hornemann, L. H. Hurley, M. K. Speedie, and H. G. Floss, *J. Am. Chem. Soc.*, **93**, 3029 (1971).
(3) M. K. Speedie, U. Hornemann, and H. G. Floss, *J. Biol. Chem.*, **250**, 7819 (1975).
(4) L. Zee, U. Hornemann, and H. G. Floss, *Biochem. Physiol. Pflanzen (Jena)*, **168**, 19 (1975).
(5) L. Mascaro, Jr., R. Hörhammer, S. Eisenstein, L. K. Sellers, K. Mascaro, and H. G. Floss, *J. Am. Chem. Soc.*, **99**, 273 (1977).
(6) For a review see H. G. Floss and M.-D. Tsai, *Adv. Enzymol.*, **50**, 243 (1979).
(7) J. W. Cornforth, J. W. Redmond, H. Eggerer, W. Buckel, and C. Gutschow, *Nature (London)*, **221**, 1212 (1969); *Eur. J. Biochem.*, **14**, 1 (1970).
(8) J. Lüthy, J. Retey, and D. Arigoni, *Nature (London)*, **221**, 1213 (1969).

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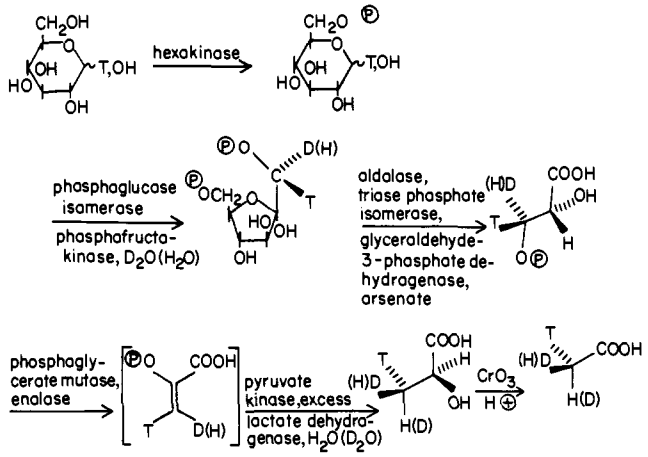
Table I. Isotope Ratios and *F* values of Substrates and Products Carrying Chiral Methyl Groups

	$^3H/^{14}C$ ratio	*F* value	methyl group confign	$^3H/^{14}C$ ratio	*F* value	methyl group confign
starting acetate	2.6	28	*S*	3.1	68	*R*
	(7.0)	(31)		(7.4)	(71)	
methionine	2.3		*R*	3.2		*S*
	(7.0)			(7.1)		
acetate from indolmycenic acid	2.4	35	*S*	2.7	64	*R*
indolmycenic acid	2.4		*S*	3.2		*R*
acetate from methylamine	2.4	64	*R*	3.4	37	*S*
indolmycin	2.5		*S* (*N*-Me)	3.2		*R* (*N*-Me)
acetate from indolmycin	2.2	37	*S*	2.9	65	*R*
	(6.5)	(39)		(7.0)	(63)	
indolmycin	2.5		*S* (*C*-Me)	3.2		*R* (*C*-Me)
	(7.1)			(6.9)		

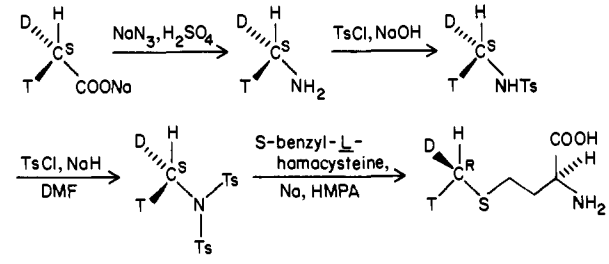
Scheme I. Enzymatic Synthesis of Chiral Acetate



Arigoni,[8] using essentially the procedure described by Cornforth, Eggerer, et al.[6,7] Acetate was converted enzymatically into acetyl-coenzyme A which was condensed with glyoxylate in a reaction catalyzed by malate synthase. The resulting malate was analyzed for the distribution of tritium between the two diastereotopic hydrogen positions at C-3 by incubation with fumarase, which stereospecifically equilibrates the *pro*-3*R* hydrogen of malate with solvent protons. A kinetic isotope effect in the malate synthase reaction results in an asymmetric tritium distribution at C-3 of malate if the acetate is chiral, such that chirally pure *R* acetate gives malate which retains 79% of the tritium in the fumarase reaction, whereas chirally pure *S* acetate produces malate which retains 21% of its tritium upon incubation with fumarase. The percentage of tritium retention in the fumarase reaction which characterizes the configuration and chiral purity of a chiral acetate sample is referred to as the *F* value.

The starting material for the synthesis of methionine was chiral [2-$^2H_1$,2-$^3H_1$]acetate, which was prepared by the general route shown in Scheme I. The crucial step in this sequence, the one in which the chiral methyl group is generated, is the pyruvate kinase reaction, which has been shown by Rose[9] to involve protonation on the si face at C-3 of phosphoenolpyruvate. Preparation of (*R*)-[2-$^2H_1$,2-$^3H_1$]acetate involved conversion of [1-^3H]glucose in a single incubation[10] into (3*S*)-phospho[3-^3H]glycerate (PGA), which was further converted into (3*R*)-[3-$^2H_1$,3-$^3H_1$]acetate in D_2O as the medium. The oxidation of the lactate to acetate was carried out under mild conditions[11] which do not lead to any significant racemization. In the first synthesis the *S* isomer of chiral acetate was prepared analogously from (3*R*)-phospho[3-^3H]glycerate obtained in a single incubation from [1-^3H]-mannose.[11] However, since the yield of (3*R*)-[3-^3H]PGA from

Scheme II. Synthesis of Methionine Carrying a Chiral Methyl Group



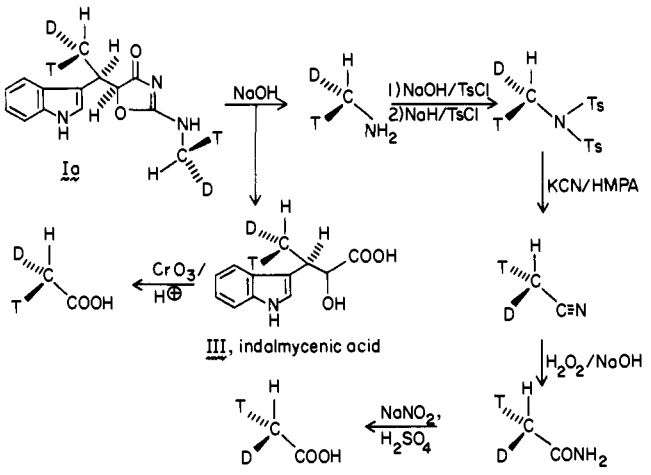
mannose is invariably much lower than that of the 3S isomer from glucose, we subsequently prepared (S)-[2-$^2H_1$,2-$^3H_1$]acetate from [1-^3H]glucose via (3*S*)-phospho[3-$^2H_1$,3-$^3H_1$]glycerate as shown in Scheme I. Deuterium was in this case introduced by conducting the phosphoglucose isomerase reaction under equilibrium conditions in D_2O, and the later conversion of PGA into lactate was done in H_2O. The overall yields of acetate from glucose are ~25%. The *F* values of the acetate samples from two syntheses were 71 and 68 for the *R* isomer and 31 and 28 for the *S* isomer. Thus, the acetate has a chiral purity (enantiomeric excess) of ~70%. The pyruvate kinase reaction inherently involves some deprotonation/reprotonation of the pyruvate before its release from the enzyme,[12] accounting for the somewhat low chiral purity of the acetate.

Conversion of the chiral acetate into methionine followed the reaction sequence outlined in Scheme II. The Schmidt reaction, used to cleave the carbon–carbon bond of acetate with conversion of the methyl group into methylamine, is known to proceed with predominant or complete retention of configuration.[13] Substitution of the nitrogen with two tosyl groups converted the amino function into a sufficiently good leaving group[14,15] to use the resulting *N*-methylditosylimide as an electrophile in the alkylation of the homocysteine *S* anion. This alkylation can be expected to proceed by an S_N2 mechanism and thus to involve inversion of configuration at the methyl group. The only plausible alternative, racemization, is ruled out by the subsequent recovery of chiral acetate in the degradations of the products from the enzymatic transfer of the methyl group of these methionine samples. As shown in the first two columns of Table I, the conversion of the [2-^{14}C,2-$^2H_1$,2-$^3H_2$]acetates into methionines proceeded without significant change in the $^3H/^{14}C$ ratios, indicating that no appreciable hydrogen exchange had occurred in this reaction sequence.

The two diastereomeric samples of L-methionine carrying the chiral methyl group (*R* isomer, 23 μCi; *S* isomer, 9.6 μCi) were fed to shake cultures of *S. griseus* (ATCC 12648) as described

(9) J. A. Rose, *J. Biol. Chem.*, **245**, 6052 (1970).
(10) H. G. Floss, D. K. Onderka, and M. Caroll, *J. Biol. Chem.*, **247**, 736 (1972).
(11) H. Simon and H. G. Floss, "Bestimmung der Isotopenverteilung in markierten Verbindungen", Springer-Verlag, Berlin, 1967, p 50.
(12) J. L. Robinson and J. A. Rose, *J. Biol. Chem.*, **247**, 1096 (1972), and references therein.
(13) Cf. E. L. Eliel, "Stereochemistry of Carbon Compounds", McGraw-Hill, New York, 1962, p 119.
(14) P. J. DeChristopher, J. P. Adamek, G. D. Lyon, J. J. Galante, H. E. Haffner, R. J. Boggio, and R. J. Baumgartner, *J. Am. Chem. Soc.*, **91**, 2384 (1969).
(15) N. H. Anderson and H.-s. Uh, *Synth. Commun.*, **2**, 292 (1972).

Scheme III. Degradation of Indolmycin and Indolmycenic Acid to Recover the *C*- and *N*-Methyl Groups as Acetate



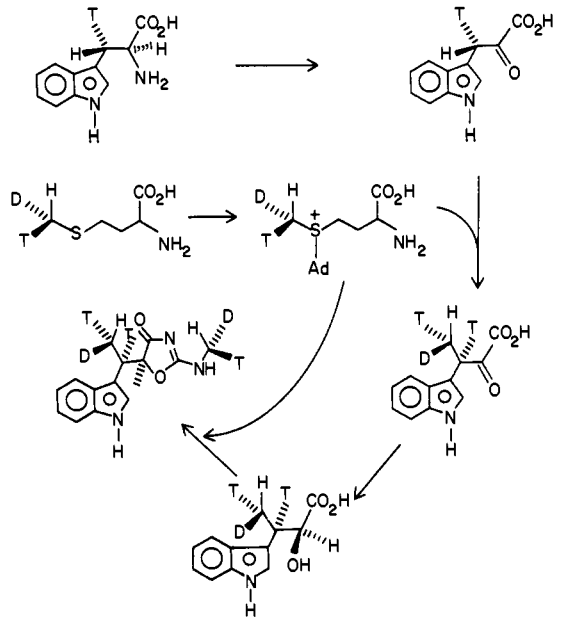
earlier,[2] and from each culture both the indolmycenic acid (III) and the indolmycin were isolated and purified as previously described.[2] As indicated in Table I there was no change in the $^3H/^{14}C$ ratios in going from methionine to III and Ia. The incorporations were 9.6 and 19.8% into indolmycin and 34.3 and 34.4%, respectively, into indolmycenic acid.

The final task, degradation of III and Ia to recover the methyl groups in the form of acetate, was carried out as shown in Scheme III. Excision of the *C*-methyl group from III and Ia, in the latter case after hydrolysis to III, by Kuhn–Roth oxidation was straightforeward. Earlier work[2] had shown that formation of acetic acid under these conditions occurs without loss of tritium from the methyl group, and the methyl group of acetate should have the same configuration as the *C*-methyl group in III or Ia. The *N*-methyl group of Ia was recovered as methylamine in the alkaline hydrolysis of the antibiotic. Conversion into the *N*,*N*-ditosylimide as in the synthesis of methionine followed by displacement with cyanide gave acetonitrile. The latter was converted into acetic acid by alkaline hydrogen peroxide oxidation followed by diazotation of the acetamide, a reaction sequence which avoids the hydrogen exchange and racemization at the α position generally encountered in the hydrolytic conversion of the nitrile into the acid.[16] In this degradation sequence inversion of configuration of the methyl group occurs in the cyanide displacement step; hence, the acetate from this degradation will have the opposite configuration as the *N*-methyl group of Ia.

The data in Table I indicate that all the degradations occur without any significant loss of tritium from the methyl group. The chirality analyses of the acetate samples obtained from the *C*-methyl groups of Ia and III reveal that these have the same configurations as the respective acetates used as starting materials in the synthesis of the corresponding methionines. Since the methionine synthesis involves one inversion of configuration, it follows that the enzymatic transfer of the methyl group to the acceptor carbon must allso have occurred with inversion of configuration. The acetates obtained from the *N*-methyl group of Ia have the opposite configuration as the starting acetates. In this case the chemical reaction steps involve two inversions of configuration, one in the synthesis of methionine and one in the conversion of methylamine into acetate. Hence, again the enzymatic transfer of the methyl group to nitrogen must have occurred in an inversion mode. A 20–40% decrease in the chiral purity of the methyl groups is observed in the overall conversion sequence, a remarkably small degree of racemization considering the number of steps involved. This partial racemization may take place during the enzymatic transfer of the methyl group; however, this seems unlikely since it is not very probable that two different enzymes transfer methyl groups to two different acceptors with the same degree of racemization. More likely the partial racemization has occurred in one of the chemical reaction steps. The two reactions that are most suspect are the Schmidt reaction and the displacement reactions of the methylditosylimine. The fact that the acetates from methylamine and the acetates from the Kuhn–Roth oxidations have the same degree of chiral purity suggests that the displacement reactions are completely stereospecific; hence, the partial racemization has most likely occurred in the Schmidt reaction.[17]

(16) D. Arigoni, F. Lynen, and J. Retey, *Helv. Chim. Acta*, **49**, 311 (1966).

Scheme IV. Stereochemical Course of Indolmycin Biosynthesis in *Streptomyces griseus*.



Discussion

The work presented in this paper outlines a general approach to the study of the steric course of one-carbon transfer reactions mediated by (*S*)-adenosylmethionine-dependent methyl transferases. The results obtained demonstrate that two such transfer reactions, one to the β carbon of indolepyruvate and one to the amino group of *N*-demethylindolmycin (Ib), occur with inversion of configuration at the methyl group. Methyl transfer in an inversion mode has also been found in the catechol–O-methyl transferase reaction[18] and in several examples investigated in Arigonis laboratory.[6] Deuterium and carbon-13 isotope effect studies on model systems[19,20] and on catechol–O-methyl transferase[21] indicate a tight S_N2 transition state for the transfer of the methyl group. This means that any single transfer of the methyl group must occur with inversion of configuration. The stereochemical finding of net inversion in both the C- and the N-methylation studied here, assuming the same transition state, indicates that the overall enzymatic process involves an uneven number of transfers of the methyl group, in all likelihood a single transfer. This would rule out a process in which the methyl group is first transferred from (*S*)-adenosylmethionine to a group on the enzyme and from there to the acceptor carbon or nitrogen.

Together with our earlier finding[4] that, in the C-methylation of IIa, a hydrogen at the β position of the substrate is replaced by the methyl group in a retention mode, the present findings define the stereochemical course of the methylation reactions in the biosynthesis of Ia as illustrated in Scheme IV. Hydrogen

(17) H. L. Holland, M. Castillo, D. B. MacLean, and I. D. Spenser, *Can. J. Chem.*, **52**, 2818 (1974).
(18) R. W. Woodard, M.-D. Tsai, J. K. Coward, and H. G. Floss, manuscript in preparation.
(19) J. Mihel, J. O. Knipe, J. K. Coward, and R. L. Schowen, *J. Am. Chem. Soc.*, **101**, 4349 (1979).
(20) C. H. Gray, J. K. Coward, K. B. Schowen, and R. L. Schowen, *J. Am. Chem. Soc.*, **101**, 4351 (1979).
(21) M. F. Hegazi, R. T. Borchardt, and R. L. Schowen, *J. Am. Chem. Soc.*, **101**, 4359 (1979).
(22) Reference 11, p 12.

replacement by CH_3 in a retention mode in the C-methylation implies that the sulfur of (*S*)-adenosylmethionine must bind to the enzyme in close proximity to a base group in the active site which mediates the proton abstraction from C-β. If this base is in the unprotonated form when (*S*)-adenosylmethionine binds, charge neutralization between the base and the positively charged sulfur is likely to interfere with the catalytic mechanism. It would therefore seem likely that the proton abstraction from C-β (enolization of IIa) occurs before (*S*)-adenosylmethionine binds. The alternative, transfer of the methyl group of (*S*)-adenosylmethionine to the enzyme and release of (*S*)-adenosylhomocysteine from the enzyme before IIa binds (ping–pong mechanism) or enolizes, is ruled out by the stereochemical results of the present study.

Experimental Section

General Materials and Methods. Organic chemicals were purchased from Aldrich Chemical Co., enzymes and biochemicals from Sigma, culture media ingredients from Difco, and radioactive compounds from Amersham-Searle and New England Nuclear Corp. All materials were used without further purification.

Radioactive samples were counted in Bray's solution or Aquasol (Beckman) in a Beckman LS-100, LS-250, or 7000 scintillation spectrometer. Counting efficiencies and the spillover of tritium into the ^{14}C channel were determined by interval standardization. Radioactivity on chromatograms was located by scanning in a Packard Model 7201 radiochromatogram scanner. TLC separations were carried out on E. Merck precoated silica gel GF-254 plates and paper chromatography on Whatman No. 1 (analytical) and 3 MM (preparative) papers. Evaporation in vacuo refers to solvent removal on a Büchi rotary evaporator at or below 40 °C.

The chirality of the acetate samples was determined by the procedure described earlier.[6,7]

(3*S*)-Phospho[3-$^2H_1$,$^3H_1$]glycerate (PGA). The following reagents were placed in a 15-mL centrifuge tube at 4 °C: 1600 μL of H_2O, 750 μL of 0.2 M Tris-HCl (pH 8.2), 120 μL of 0.1 M $MgCl_2$, 18 μL of ATP (48 mg/mL, 1.5 μmol), 7.3 μL of 0.1 M glucose (0.73 μmol), and 500 μL of [1-$^3H_1$]glucose (3.75 Ci/mM, 1 mCi, 0.267 μmol). The tube was placed in a water bath at 37 °C and the reaction was initiated by the addition of 10 U of hexokinase (E.C. 2.7.1.1, 25 μL). After incubation for 15 min, the reaction was stopped by heating at 100 °C for 2 min. Chromatography confirmed complete conversion of glucose to glucose 6-phosphate. The mixture was lyophilized and then dissolved in D_2O. This process was repeated three times. The pH of the reaction mixture in 1 mL of D_2O was adjusted to 7.8–8.0 with ~50 μL of 0.5 N NaOD in D_2O. An aqueous suspension of 10 U of phosphoglucose isomerase (E.C. 5.3.1.9, 5 μL) was added and the solution incubated for 18 h at 37 °C. Chromatography confirmed conversion of glucose 6-phosphate into fructose 6-phosphate. Fructose 6-phosphate kinase (E.C. 2.7.1.11, 15 U, 15 μL) and an additional 10 U of phosphoglucose isomerase (5 μL) were added and the mixture was incubated at 37 °C for 3 h with additions of 5 μL of ATP (48 mg/mL) in D_2O at 15, 30, 60, 90 min.

The reaction was stopped by heating at 100 °C for 2 min. The presence of fructose 1,6-diphosphate was confirmed by paper chromatography. To the above were added 200 μL of NAD^+ (5 μmol/100 μL), 10 μL of 1 M $NaHAsO_4$, 10 U of aldolase (E.C. 4.1.2.13, 100 μL), 26 U of triose *P*-isomerase (E.C. 5.3.1.1, 1 μL), and 14 U of glyceraldehyde *P*-dehydrogenase (E.C. 1.1.1.8, 20 μL) and the mixture was incubated for 3 h at 37 °C. The reaction was stopped by heating 2 min at 100 °C and the mixture was chromatographed on citrate-washed[10] Whatman 3 MM paper with *n*-BuOH/AcOH/H_2O (2:1:1) as the solvent. The PGA zone was eluted from the paper with water, diluted with carrier material to a specific activity of 100 mCi/mmol, and used immediately for the next step. The yield, 8.8×10^8, dpm, = 39.6%.

(3*R*)-Phospho[3-$^3H_1$]glycerate. A modification of the above procedure was used in which after the conversion of glucose into glucose 6-phosphate, the following reagents were added to the reaction mixture in a 15-mL centrifuge tube: 200 μL of 50 mM NAD^+, 10 μL of 1 M $NaHAsO_4$, 20 U of phosphoglucose isomerase, 16 U of fructose 6-phosphate kinase, 10 U of aldolase, 25 U of triose phosphate isomerase, and 13 U of glyceraldehyde 3-phosphate dehydrogenase. The mixture was incubated at 37 °C for 3 h with 5-μL aliquots of ATP (48 mg/mL) in H_2O being added at 15, 30, 60, and 90 min. The PGA was isolated and diluted as described above. The yield was 43.2%.

(3*S*)[3-$^2H_1$,$^3H_1$]Lactate. The following reagents were placed in a 3-mL quartz cuvette: 300 μL of 0.5 M triethanolamine (TEA)–HCl buffer (pH 7.6), 150 μL of 0.1 M $MgSO_4$, 15 μL of 2,3-diphosphoglycerate (8 mg/mL), 200 μL of 0.1 M ADP, 75 μL of 2 M KCl, (3*S*)-[3-$^2H_1$,$^3H_1$]PGA (enough carrier 0.1 M PGA to give 10 μmol of PGA in the assay), and H_2O to a volume of 2940 μL. The pH of the solution was adjusted to 7.4–7.6 with 1.0 N NaOH, and 30 μL of a solution of enolase (E.C. 4.2.1.11, 10 mg/mL) in H_2O was added to the cuvette. The absorbance at 340 nm was adjusted to zero and 60 μL of 0.1 M NADH was added. Twenty units of pyruvate kinase (E.C. 2.7.1.50), 60 U of lactate dehydrogenase (E.C. 1.1.1.27), and 30 U of phosphoglycerate mutase (E.C. 2.7.5.3) were dialyzed for two 30-min periods against 20 mL of 0.05 M TEA–HCl buffer (pH 7.6). The dialyzed enzyme solution was added to the cuvette and the change in A_{340} monitored at 37 °C. When the rate of change slowed, 15 μL of 0.1 M NADH was added. After the reaction stopped, the mixture was transferred to a 15-mL centrifuge tube, heated at 100 °C for 2 min, and then chromatographed on citrate-washed Whatman 3 MM paper with EtOH/NH_4OH/H_2O (80:4:17) as solvent. The lactate was eluted from the chromatogram with water. The yield was 76.1%.

(3*R*)-[3-$^2H_1$,$^3H_1$]Lactate. The same procedure as above was used, except as follows. The (3*R*)-[3-$^3H_1$]PGA solution was lyophilized, dissolved in 1 mL of D_2O, and lyophilized again. All of the reagents except the enzymes were mixed and then repeatedly lyophilized, taken up in D_2O, and lyophilized again. The enzymes were dialyzed for two 30-min periods against 20 mL of TEA–DCl buffer (pH 7.6) in D_2O. All components were then mixed as solutions in D_2O. The rest of the procedure was identical with that described for the 3*S* isomer. The yield was 74.5%.

(2*R*)- and (2*S*)-[2-$^2H_1$,$^3H_1$]acetate. A 100-mL base-washed recovery flask was charged with the lactate sample and cooled to 0 °C, and 30 mL of cold oxidizing solution (153 mg of $K_2Cr_2O_7$, 24 mL of concentrated H_2SO_4, and water to 100 mL) was added. The mixture was refluxed for 1 h under an efficient condenser, during which time it remained yellow for at least 30 min, and then steam-distilled. A total of 100 mL of distillate was collected by adding 25-mL portions of water to the distillation flask as it became almost dry. The distillate was adjusted to pH 10 with 1 N NaOH and evaporated to dryness. Yields of acetate ranged from 75 to 85%. The acetate samples were mixed with sodium [2-^{14}C]acetate to give the desired $^3H/^{14}C$ ratios.

(*R*)- and (*S*)-[^{14}C,$^2H_1$,$^3H_1$]methylamine. A 50-mL three-neck flask, equipped with a magnetic stirring bar, a bent solid addition tube, a pressure-equalizing addition funnel, and a N_2 inlet valve was charged with thoroughly dried (*S*)-[2-^{14}C,$^2H_1$,$^3H_1$]acetate (85.7 mg, 1.05 mmol, 0.96 mCi, $^3H/^{14}C$ = 2.55) and cooled to 0 °C while 1.7 mL of 100% H_2SO_4 (1.2 mL of concentrated H_2SO_4 + 0.5 mL of fuming H_2SO_4) was added slowly. After addition of the acid, the mixture was allowed to warm to room temperature and then heated to 65 °C, and sodium azide (134 mg, 2.06 mmol) was added from the solid addition tube in small portions, allowing cessation of nitrogen evolution before the next addition. The addition required 1 h and the mixture was heated for a further 5 h. The addition tube was replaced with a vacuum distillation apparatus, the receiver flask was charged with 50 mL of 2.5 N HCl, and the vacuum adapter outlet was connected to a gas trap also containing 2.5 N HCl. H_2O (3 mL) was added and the mixture was steam distilled under a strong flow of N_2. Two further 25 mL portions of water were added and the mixture was steam distilled. The solutions of HCl containing the amine were combined and evaporated to give (*S*)-[^{14}C,$^2H_1$,$^3H_1$]methylamine hydrochloride (50 mg, 0.74 mmol = 73.9%, 0.71 mCi, $^3H/^{14}C$ = 2.4). In the same fashion, sodium (*R*)-[2-^{14}C,$^2H_1$,$^3H_1$]acetate (85 mg, 1.0 mmol, 0.64 mCi, $^3H/^{14}C$ = 3.1) gave (*R*)-[^{14}C, $^2H_1$,$^3H_1$]methylamine hydrochloride (48.5 mg, 0.72 mmol = 71.9%, 0.46 mCi, $^3H/^{14}C$ = 2.9).

(*R*)- and (*S*)-*N*-[^{14}C,$^2H_1$,$^3H_1$]methyl-*N*,*N*-di-*p*-toluenesulfonimide. To a frozen solution of (*S*)-[^{14}C,$^2H_1$,$^3H_1$]methylamine hydrochloride (67.5 mg, 1 mmol, 0.71 mCi, $^3H/^{14}C$ = 2.4) in 6 mL of 10% sodium hydroxide was added *p*-toluenesulfonyl chloride (285.9 mg, 1.5 mmol). The flask was sealed with a rubber septum and heated at 100 °C for 2 h. After cooling, 3 mL of 10% NaOH was added, and the flask was heated to 100 °C for 2 h. The reaction mixture was cooled to 0 °C and 10% HCl was added until a white precipitate formed. The pH was adjusted to 7 and the mixture was evaporated to dryness to yield crude *N*-methyl-*p*-toluenesulfonamide (176 mg, 0.67 mCi, $^3H/^{14}C$ = 2.4). The latter was dissolved in 30 mL of dry DMF. Then 42 mg (0.88 mmol) of NaH as a 50% suspension in oil was added slowly with cooling to form the sodium salt of the sulfonamide. After 1 h of stirring, *p*-toluenesulfonylchloride (286 mg, 1.5 mmol) was added and the mixture was stirred for 12 h and then poured into 50 mL of water. The aqueous phase was extracted with chloroform, washed with cold saturated Na_2CO_3 solution, dried over $MgSO_4$ and evaporated to dryness (yield 303 mg, 0.89 mmol, 0.63 mCi = 89.3%, $^3H/^{14}C$ = 2.3). Similarly, (*R*)-[^{14}C,$^2H_1$,$^3H_1$]methylamine hydrochloride (67.5 mg, 1.0 mmol, 0.46 mCi, $^3H/^{14}C$ = 2.9) gave the title sulfonimide of *R* configuration (158 mg, 0.46 mmol, 0.21 mCi = 46.4%, $^3H/^{14}C$ = 2.8).

(2*S*,*methyl*-*S*)- and (2*S*,*methyl*-*R*)-[*methyl*-^{14}C,$^2H_1$,$^3H_1$]methionine. To (*S*)-benzyl-L-homocysteine (208 mg, 1 mmol) in 45mL of liquid NH_3 was added Na in small pieces until a blue color was maintained for 20 min. The liquid NH_3 was allowed to evaporate under a stream of argon.

To the solid was added 10 mL of dry HMPA, the mixture was frozen and evacuated, and argon was allowed to leak back in. This process was repeated three times and, before the final thawing, the *S* isomer of the sulfonimide from the previous step (0.63 mCi) was added under a strong flow of argon. The mixture was allowed to come to room temperature and then heated at 80 °C for 16 h. After it cooled to 0 °C, 2 mL of H_2O was added, and the pH was adjusted to 7 with HCl. The solution was loaded onto a column of Dowex 50W × 8, 100-200 mesh. The column was washed with 30 mL of H_2O and eluted with 10% NH_4OH. All ninhydrin positive fractions were combined, concentrated under vacuum, and subjected to preparative TLC with *n*-BuOH/HOAc/H_2O (4:1:1) as solvent. The methionine (R_f 0.4) was eluted from the silica with water, concentrated to dryness, and recrystallized from acetic acid (yield 48.7 mg, 0.23 mCi = 36.7%, $^3H/^{14}C$ = 2.3). The *methyl-S* isomer of methionine (31 mg, 0.1 mCi = 44.9%, $^3H/^{14}C$ = 3.2) was prepared in the same manner from the *R* isomer of the sulfonimide (0.21 mCi).

Conversion of Methionines to Ia and III. Fermentations of *S. griseus* strain ATCC 12648 and the isolation of Ia and III were carried out as described earlier.[2] Feeding of 23 μCi of the *methyl-R* isomer of methionine ($^3H/^{14}C$ = 2.3) gave 2.2 μCi of Ia ($^3H/^{14}C$ = 2.5) and 7.89 μCi of III ($^3H/^{14}C$ = 2.4); 9.6 μCi of the *methyl-S* isomer ($^3H/^{14}C$ = 3.2) produced 1.9 μCi of Ia ($^3H/^{14}C$ = 3.2) and 3.3 μCi of III ($^3H/^{14}C$ = 3.2).

Degradation of Ia and III. Indolmycin from the two feeding experiments was hydrolyzed with base[2] to give indolmycenic acid and methylamine, isolated as the hydrochloride. Both the indolmycenic acids from this hydrolysis and the ones isolated directly from the cultures were subjected to Kuhn-Roth oxidation[22] to give acetic acid from the *C*-methyl group.

The two samples of methylamine hydrochloride from the hydrolysis of Ia were converted into the *N*-methyl-*N*,*N*-di-*p*-toluenesulfonimide as described above. The latter, 100 mg of KCN, and 5 mL of dry HMPA were quickly frozen in a 25-mL single-neck recovery flask equipped for magnetic stirring. The flask was connected to a second 25-mL recovery flask containing 1 mL of H_2O and a magnetic stirring bar via a vacuum bridge. Both flasks were cooled to -78 °C and the system was evacuated and closed. The flask containing the reaction mixture was then heated with stirring at 85 °C while the second flask was cooled to -78 C. The heating was continued for 72 h. The reaction flask was then replaced with a clean flask and to the cold flask containing the water and acetonitrile were added 3 mL of 30% H_2O_2 and 0.1 mL of 6 N NaOH. The mixture was heated at 50 °C for 6 h and then evaporated to dryness to yield acetamide (yield 19-23% based on methylamine).

The acetamide was dissolved in 1 mL of H_2O and cooled to 0 °C. Cold 5 N H_2SO_4 (2 mL) was added slowly with stirring, followed by 0.4 g of $NaNO_2$ in 1 mL of H_2O. After 1 h at 0 °C, the mixture was stirred for 5 h at room temperature. The acetate was isolated by steam distillation as described above. The yields in this step were quantitative.

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Enthalpy Changes Accompanying Hydrolysis of 3-(2-Furyl)acryloylimidazole by α-Chymotrypsin

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Abstract: A calorimetric analysis of the α-chymotrypsin-mediated hydrolysis of 3-(2-furyl)acryloylimidazole at pH 7.85 is presented and compared with the nonenzymic heat of hydrolysis. The enzyme-mediated hydrolysis is characterized by an apparent pK_a for acylation of 6.60 and a deacylation pK_a of 7.55. Activation enthalpy changes of 7.8 ± 1.3 and 15.0 ± 0.4 kcal/mol were determined for acylation (k_2) and deacylation (k_3), respectively. An enthalpy change of 0.04 ± 0.85 kcal/mol for Michaelis complex formation was also determined from the temperature dependence of K_s. Heats of acylation (-10.1 ± 0.2 kcal/mol) and deacylation (-1.2 ± 0.55 kcal/mol) corrected for buffer ionization and product ionization heats were separately determined by making use of the large rate difference between acylation and deacylation ($k_2 = 1000k_3$) which exists at pH 7.85. The sum of these corrected enthalpy changes (-11.3 ± 0.6 kcal/mol) agrees very well with the enthalpy change observed for nonenzymic hydrolysis of furylacryloylimidazole (-11.3 ± 0.5 kcal/mol) and validates the approach used.

Introduction

The monomolecular and bimolecular reactions (elementary events) which characterize an enzyme catalytic sequence are of major interest in enzymology since they contain the factors responsible for catalysis. These elementary events are composed of collections of part processes such as conformational changes, noncovalent binding of reactants and products to enzyme, desolvation of active site and reactants, solvation of products and active site, covalent bond rearrangements, etc.[1] To add to this complexity, perturbations of ionizable groups on the enzyme (and also substrates and products) frequently result in hydrogen ion uptake or release being coupled to the monomolecular or bimolecular event.

Since thermodynamic parameters (e.g., ΔG, ΔH, ΔS) which describe an elementary event are composed of contributions from various part processes, they are not easy to interpret in detail. The evaluation of any part process in thermodynamic terms is one of the single most difficult problems in enzymology and only through the use of a variety of techniques does there appear much hope of quantitating the more prominent terms in an elementary event. We believe that calorimetric measurements hold great promise for defining enthalpy changes of such monomolecular and bimolecular steps and under favorable conditions could lead to dissection of an event into its component part processes. This objective cannot readily be realized since calorimetry has historically been used to measure equilibria rather than kinetic events, and, consequently, only a very limited number of techniques have been developed for application to catenary chain mechanisms. Clearly, the first goals must be to establish procedures for measuring enthalpy changes for elementary events and to place such measurements on a firm foundation.

The work reported here deals with several aspects of measuring the enthalpy changes for hydrolysis of 3-(2-furyl)acryloylimidazole (FAI)[2] as mediated by α-chymotrypsin (EC 3.4.4.5). These aspects include (1) the separation and calorimetric measurements

(1) Jencks, W. P. *Adv. Enzymol.* **1976**, *43*, 219-409.

(2) The abbreviations used: FA-Ct, furylacryloylchymotrypsin; FAH, protonated furylacrylic acid; FA^-, unprotonated furylacrylic acid; FAI, 3-(2-furyl)acryloylimidazole.

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