Proton Exchange on Carbons 2 and 3 of Serine during Their Conversion into Methyl Groups of Methionine and Thymine in *Escherichia coli*†

Robert H. White

ABSTRACT: The conversion of the C-2 and C-3 carbons of serine and their attached protons into the methyl groups of methionine and thymine was studied in vivo with exponentially growing cultures of *Escherichia coli*. This was accomplished by measuring the distribution of deuterium incorporated into the methyl groups of thymine and methionine when cells were grown in the presence of deuterated serines. The results show that both the C-2 and C-3 carbons of serine are converted into the methyl groups of these compounds. The extent of incorporation of deuterium from both the C-2 and C-3 carbons of serine, however, was found to be different for the thymine and methionine methyl groups. In addition, partial exchange of carbon-bonded deuteriums was observed to occur from both of these carbon sources. Evidence is presented that demonstrates that the exchange of the C-3 deuterium most likely results from the reversible conversion of 5,10-methylene-H_4folate to 5,10-methenyl-H_4folate catalyzed by 5,10-methylene-H_4folate dehydrogenase. The specific enzymatic site for the exchange of the C-2 protons was not identified but was confirmed by the presence of glycine in the cell containing less than the expected abundance of a single deuterium. A possible explanation for the difference in the deuterium incorporation into these two methyl groups is discussed.

The pathway for the conversion of the C-2 and C-3 carbons of serine into the methyl groups of methionine and thymine is well established and is outlined in Figure 1 (Harvey & Dev, 1975). All of the individual reactions shown have been studied in sufficient detail that specific information about the exchange of carbon-bonded hydrogens originating from the serine is known. In the first reaction, serine is reversibly cleaved to form glycine and 5,10-methylene-H_4folate. The reaction is known to proceed as shown in Figure 1 with the retention of the C-2 proton of L-serine in the 2*R* position of the glycine (Jordan & Akhtar, 1970) and the retention of the original serine C-3 protons in the methylene group of the 5,10-methylene-H_4folate (Tatum et al., 1977). Additional 5,10-methylene-H_4folate can be supplied by the glycine cleavage reaction. This reversible reaction decomposes glycine with the formation of 1 mol each of 5,10-methylene-H_4folate, ammonia, and CO_2 as shown in reaction b, Figure 1. Since both glycine protons are retained during the glycine cleavage reaction (Kochi & Kikuchi, 1974), any serine metabolized by this pathway will retain its C-2 proton in the resulting 5,10-methylene-H_4folate.

The methylene group of the 5,10-methylene-H_4folate that results from these two reactions is then converted by separate pathways into the methyl group of methionine or the methyl group of thymine. In the first pathway, that leading to methionine, the 5,10-methylene-H_4folate is reduced in a $FADH_2$-dependent reaction to 5-methyl-H_4folate. The enzyme that catalyzes this reaction has been partially purified from *Escherichia coli* (Katzen & Buchanan, 1965) and has been shown to incorporate only one proton from water into the methyl group of the resulting 5-methyl-H_4folate (Kisliuk, 1963; Cathou & Buchanan, 1963). The methyl group of 5-methyl-H_4folate is then transferred to homocysteine, resulting in the formation of methionine. In *E. coli*, this reaction can occur by either a B_{12}-dependent or a B_{12}-independent pathway (Blakely, 1969). Current evidence would indicate that, regardless of which pathway is used, all of the methyl protons of 5-methyl-H_4folate are transferred into methionine intact (Kisliuk, 1963; Cathou & Buchanan, 1963).

In the pathway leading to thymine, the methyl group of thymine originates directly from 5,10-methylene-H_4folate by the simultaneous transfer and reduction of the methylene group to C-5 of deoxyuridine monophosphate, resulting in the formation of thymidylic acid. This reaction is known to proceed with the retention of the methylene protons in the methyl group (Lomax & Greenberg, 1967). The additional proton of the methyl group arises from the A side of the nicotinamide ring of NADPH via the reduced pteridine ring of the 5,10-methylene-H_4folate without solvent exchange (Blakley et al., 1963).

On the basis of the above discussion, one would expect both the C-2 and C-3 groups of deuterated serine and their attached deuterium atoms to be incorporated into the methyl groups of both methionine and thymine without any loss of label. Thus, we would expect that the distribution of deuterium found in vivo on C-2 and C-3 of serine would also be reflected in the deuterium incorporated into the methyl groups of both thymine and methionine. However, as reported in this paper, we find less than the expected deuterium content in the methyl groups of both the methionine and thymine produced by cells grown with deuterated serine. In addition, the extent of this deuterium deficiency was found to be different for the thymine methyl group and the methionine methyl group. Evidence is also presented showing that this deuterium deficiency is the result of exchanges occurring at two separate sites. The first site most likely involves the reversible conversion of 5,10-methylene-H_4folate into 5,10-methenyl-H_4folate catalyzed by the enzyme 5,10-methylene-H_4folate dehydrogenase. The second site involves exchange of the glycine C-2 protons, which are shown to supply ~30% of the 5,10-methylene-H_4folate used for the synthesis of the thymine and methionine methyl groups. The reason for this exchange is unknown but some process in the cell generates some glycines with two deuteriums.

Experimental Procedures

Materials

DL-Serine-*2,3,3-d*$_3$ and DL-serine-*3,3-d*$_2$ (98.0 atom % ^2H) were obtained from Merck Sharp & Dohme of Canada.

† From the Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061. *Received August 19, 1982; revised manuscript received January 11, 1983.* Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for partial support of this research.

0006-2960/83/0422-1883$01.50/0

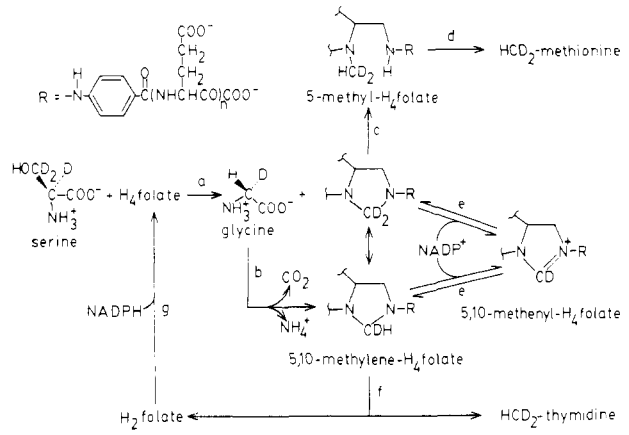

FIGURE 1: Pathways for conversion of C-2 and C-3 carbons of serine and their attached protons into methyl groups of thymine and methionine. The individual reactions are catalyzed by the following enzymes: (a) serine hydroxymethyltransferase; (b) glycine cleavage reaction; (c) 5,10-methylene-H_4folate reductase; (d) N^5-methyl-H_4folate:homocysteine transmethylase; (e) N^5,N^{10}-methylene-H_4folate dehydrogenase; (f) thymidylate synthetase; (g) H_2folate reductase.

Methods

Bacterial Strains and Growth Medium. *E. coli* K12 strain CU 331 (formerly AT 2475) *serA ara* was supplied by Dr. H. E. Umbarger of Purdue University and *E. coli* B strain WG 1143 *ser A12* was supplied by Dr. W. B. Dempsey, Veteran's Administration Hospital, Dallas, TX.

The cells were grown on 25 mL of a defined liquid medium consisting of 12.1 g of Tris, 7.7 g of KH_2PO_4, 5.3 g of NH_4Cl, 2.9 g of sodium citrate dihydrate, 4 g of glucose, 0.5 g of NaCl, 800 mg of DL-serine-*2,3,3-d*$_3$ or DL-serine-*3,3-d*$_2$, 32 mg of L-cystine, 100 mg of $MgCl_2 \cdot 6H_2O$, 44 mg of $CaCl_2 \cdot 2H_2O$, and 0.8 mg of $FeCl_3 \cdot 6H_2O$/L of distilled water. B_{12} and guanosine were added as indicated, and the pH was adjusted to 7.0 with 6 M HCl. Stock cultures of the organisms were maintained on agar slants of the same medium containing unlabeled L-serine. Cells from 12 h old slants grown at 37 °C were used to inoculate the growth medium, which was then shaken at 37 °C to the end of log-phase growth, about 8–10 h. Cells were isolated by centrifugation, boiled for 10 min in water, and recentrifuged in order to remove any unbound amino acids.

Isolation and Measurement of Deuterium Distribution in Cellular Thymine. Boiled cell pellet (~50 mg wet wt) was placed in 1 mL of 7.5 M $HClO_4$ and heated at 100 °C for 1 h. At the end of this time, 1 mL of 7.5 M KOH was added, and the resulting $KClO_4$ and precipitated carbon particles were removed by centrifugation. [This procedure is basically the method developed by Marshak & Vogel (1951) for the liberation of bases from nucleic acids.] The resulting thymine was purified by elution from a 0.6 × 6 cm column of Dowex 50-8X with 1 M HCl as described by Wall (1953). The thymine-containing peak was isolated and concentrated to dryness and its mass spectra obtained by direct probe introduction. The deuterium distribution was calculated as described below for the amino acids. Since the only protons in thymine that can be derived from serine without exchange with water are those transferred from 5,10-methylene-H_4folate during the synthesis of the methyl group (Kisliuk, 1963), the deuterium distribution in the entire molecule can be used to determine the deuterium distribution in the methylene group of 5,10-methylene-H_4folate used in the synthesis.

Measurement of Deuterium Distribution of Cellular O-, N-, and S-Bound Methyl Groups. Boiled cell pellet (2–10 mg) was placed in a small vial fitted with a rubber septum through which gas samples could be removed for head-space gas analysis. A total of 0.1 mL of HI (57%) and 0.2 mg of tin metal was added, the atmosphere exchanged with nitrogen gas, and the entire flask heated at 100 °C for 2 h. By this procedure, methyl groups attached to oxygen, nitrogen, and sulfur atoms in the cell are cleaved with the production of methyl iodide in the gas phase. GC–MS[1] analysis of the methyl iodide was used to measure the deuterium distribution in the methyl groups. Separation of the gas was performed on a $^1/_8$ in. × 6 ft Chromosorb 101 column operated at 130 °C. Isotope abundances were measured from the molecular ion of methyl iodide at m/z 142.

Measurement of Deuterium Distributions in Amino Acids. Analyses of the isotopic distributions of ^2H in the bound serine, glycine, proline, threonine, and methionine were performed by GC–MS of the *N*-trifluoroacetyl *n*-butyl ester derivatives. The preparation of these derivatives from the bound amino acids released by the 6 N acid hydrolysis of cells has been described by White (1981). The label in these bound amino acids is expected to reflect the average isotope distribution of the free amino acids that were present in the cells over the entire growth of the bacteria. This is a valid conclusion for bacterial cells due to the lack of compartmentalization in the cells and the lack of protein turnover in growing *E. coli* cells (Rotman & Spiegelman, 1954; Koch & Levy, 1955; Hogness et al., 1955).

Ion intensities were measured from ions in both the electron impact (EI) and chemical ionization (CI) spectra of the separated amino acid derivatives. Isotopic fractionation of the deuterated compounds during gas chromatography was corrected for by averaging the observed ion intensities over the entire gas chromatographic peak. MH^+ ions in the isobutane CI spectrum of the derivatives were used to measure the amount of deuterium present in the serine, threonine, and glycine. Both molecular ions and fragment ions in the 70-eV EI spectrum of the derivatives were used to measure the extent and, in a few cases, the position of isotopic incorporation. These included the m/z 61 ($CH_3SCH_2^+$), m/z 153 ($M^+ - C_4H_9COO - SCH_3$), and m/z 301 (M^+) ions in the methionine derivative, the m/z 172 ($M^+ - C_4H_7$), m/z 154 ($M^+ - OC_4H_9$), and m/z 126 ($M^+ - C_4H_9COO$) ions in the glycine derivative, and the m/z 267 (M^+) and m/z 166 ($M^+ - C_4H_9COO$) ions in the proline derivative. The origins of these fragments have been previously described (Gelpi et al., 1969; Leimer et al., 1977). All isotopic abundances reported in this paper were obtained from isotopic intensity measurements of these ions. The isotopic distributions are reported as the percent of the total molecules having the indicated number of deuterium atoms.

The reported isotopic distributions of deuterium in the derivatives were calculated from the experimentally observed isotopic ion intensities (shown in parentheses in the tables), which were corrected for the natural abundances of ^{13}C, ^{18}O, ^{33}S, ^{34}S, and ^{15}N by subtracting the experimentally observed isotopic ion intensities that were determined from unlabeled samples. This procedure has been outlined by Biemann (1962). The experimentally observed normalized ion intensities for the parent ion (100%), ion plus 1 m/z, and ion plus 2 m/z were as follows: serine (CI) m/z 354 (MH^+), 100, 17.9, 3.8; glycine (CI) m/z 228 (MH^+), 100, 13.3, 1.9; glycine (EI) m/z 172 ($M^+ - C_4H_7$), 100, 5.0; glycine (EI) m/z 154 ($M^+ - OC_4H_9$), 100, 6.2; methionine m/z 61 ($CH_3SCH_2^+$), 100, 17.2, 5.1; thymine (EI) m/z 126 (M^+), 100, 8.6, 2.8. Discrepancies between the observed ion intensities and those calculated from

[1] Abbreviation: GC–MS, gas chromatography–mass spectroscopy.

Table I: Incorporation of DL-Serine-*2,3,3-d*$_3$ into Bound L-Serine and Glycine and the Methyl Group of Bound L-Methionine by *E. coli* Cells

	distribution of ^2H in Ser *m/z* 254 (MH^+) ion				distribution of ^2H in Met *m/z* 61 ion				distribution of ^2H in Gly		
strain/mutant	$^2H_0$	$^2H_1$	$^2H_2$	$^2H_3$	$^2H_0$	$^2H_1$	$^2H_2$	$^2H_3$	$^2H_0$	$^2H_1$	$^2H_2$
E. coli B	59.8[a] (100)[b]	8.9 (36.8)	8.5 (21.4)	22.8 (41.7)	66.6 (100)	13.1 (37.2)	19.6 (43.4)	0.7 (9.6)	ND[f]		
E. coli B WG 1143 *serA*	15.4 (100)	11.5 (96.9)	18.2 (81.3)	54.9 (226.5)	29.5 (100)	27.7 (111.1)	41.2 (160.7)	1.5 (3.5)	42.0[c] (100.0)	40.8 (103.5)	17.2 (46.9)
									38.6[d] (100)	40.8 (110.6)	20.6 (58.6)
E. coli K12	56.4 (100)	10.3 (40.2)	9.0 (23.8)	24.3 (53.7)	64.3 (100)	12.9 (37.2)	22.4 (43.4)	0.4 (9.6)	64.9[e] (100)	26.8 (54.6)	8.3 (20.2)
E. coli K12 CU 331 *serA*	ND				29.6 (100)	21.8 (90.9)	47.4 (178.3)	1.2 (36.5)	48.1 (100)	37.1 (80.5)	14.8 (43.0)

[a] Numbers are the calculated molar ratio of molecules containing the indicated number of deuteriums. [b] Observed ion intensities with the nondeuterated ion assigned an intensity of 100. [c] Determined from the *m/z* 154 ion in the 70-eV spectra of glycine. [d] Determined from the *m/z* 172 ion in the 70-eV spectra of glycine. [e] Determined from the *m/z* 228 (MH^+) ion in the CI spectra of glycine. [f] ND, not determined.

the composition of the ion are apparent in several of these ions. These discrepancies result from the presence of ions with the retention or loss of a proton and do not significantly alter the final calculated isotopic distributions.

Confirmation That Deuterium Measured from the m/z 61 Ion in the Mass Spectrum of the Methionine Derivative Is an Accurate Measurement of Deuterium in the Methyl Group of Methionine. The *m/z* 61 ion in the mass spectrum of methionine contains not only the protons of the methyl group but also two protons from the C-4 of the methionine as well. Since the *m/z* 61 ion is used in this work to establish the distribution of deuterium on the methyl group, it must be shown that little (<1%) or no deuterium is incorporated at this carbon and that this ion can be used to correctly measure the deuterium distribution in the methyl group. This was confirmed by measuring the deuterium content in the *m/z* 154 ($M^+ - HSCH_3$) ion, which should still contain the C-4 protons, in the mass spectra of the methionine derivative. Since this ion contains the C-4 protons and none of the methyl protons, absence of deuterium in this ion can be used to establish the absence of deuterium on the C-4 carbon. In the experiments with the largest incorporation of deuterium in the methionine, the *m/z* 154 ion always showed less than 1% deuterium. This would indicate that little if any of C-4 deuterium was interfering with the measurement of deuterium distribution from the *m/z* 61 ion. That all of the label was present in the methyl group and was accurately measured from the *m/z* 61 ion was confirmed by comparing its measured deuterium distribution with that of the methyl iodide isolated from the cells by treatment with hydrogen iodide. All methods gave the same distributions within a 1% error. Measurement of the deuterium in the molecular ion for the methionine derivative, although less intense than the *m/z* 61 ion, also gave the same values.

Exchange of Labeled Amino Acids during Hydrolysis and Derivative Formation. So that one could check for the possible exchange of label during sample workup, glycine-*2,2-d*$_2$, serine-*2,3,3-d*$_3$, and methionine-*methyl-d*$_3$ were added to cell pellets before and after acid hydrolysis (6 M HCl, 24 h, 110 °C). The amino acids were then worked up, and the derivatives were prepared. Only glycine showed any observed exchange. This corresponded to a loss of 20% of the deuterium from each of the labeled C-2 positions.

Results and Discussion

Labeling of Intracellular Serine Pool. Deuterated serine from the medium is diluted by endogenously synthesized serine both in wild-type *E. coli* and in serine auxotrophs (Table I). The labeled serine incorporated into the cells also undergoes proton exchange as is evident by the presence of serine molecules containing one and two deuteriums. The majority of this exchange would be expected to occur at C-2 of the serine due to transamination and/or reversible exchange with unlabeled glycine via serine hydroxymethyltransferase. Chemical exchange was found not to occur during the serine isolation and derivative formation. That the exchange was occurring predominantly at C-2 was confirmed by the lack of monodeuterated serine isolated from cells grown with serine-*3,3-d*$_2$ (Table III). Therefore, in the serine auxotrophs grown on serine-*2,3,3-d*$_3$, >75% of the C-3 carbons of the cell can be labeled with two deuteriums. The 75% is calculated from the sum of the serines containing two and three deuteriums (Table I).

Labeling of Methionine and Thymine Methyl Groups. One would expect this same deuterium content in the methyl groups of methionine and thymine since the C-3 of serine is considered to be the sole source of these methyl groups in *E. coli* (Dev & Harvey, 1982). However, only about 38–46% of the methionine and thymine methyl groups are found to contain 2 deuterium atoms (Tables I and II). Furthermore, there is a significant increase in the number of methyl groups containing a single deuterium atom.

These results suggest that additional exchange of the C-3 protons of serine was occurring during the formation of these methyl groups and/or a portion of the methyl groups was being produced from the C-2 of serine which contains only one deuterium. The incorporation of a single proton from the C-2 carbon of serine into the methyl groups could easily result from the cleavage of the glycine produced in the serine hydroxymethyltransferase by the glycine cleavage reaction to produce monodeuterated 5,10-methylene-H_4folate as shown in reaction b, Figure 1.

Finally, the distribution of label in the thymine methyl group is always found to be different from that observed in the methionine methyl group (Tables II and III). Since both of these methyl groups arise from 5,10-methylene-H_4folate without exchange of the methylene group, there must be some mechanism whereby methylene-H_4folates labeled to different extents are differentially used for the production of these two methyl groups.

Involvement of Glycine Cleavage Reaction in Incorporation of C-2 of Serine into Methyl Groups of Methionine and Thymine. The glycine cleavage reaction is believed to be completely suppressed in *E. coli* grown in the presence of serine and in the absence of glycine as these cells have been (Cheeseman & Crosbie, 1966; Meedel & Pizer, 1974; Crosbie, 1966; Pizer, 1965; Dev & Harvey, 1982). However, the data herein clearly indicate that the proton on the C-2 of serine is incorporated into the methyl groups of both thymine and

Table II: Incorporation of DL-Serine-*2,3,3-d*$_3$ into Methionine, Thymine, and Glycine by the *E. coli* K12 CU 331 *serA* Mutant

	distribution of ^2H in Met *m/z* 61 ion				distribution of ^2H in Thy *m/z* 126 (M^+) ion				distribution of ^2H in Gly		
expt	$^2H_0$	$^2H_1$	$^2H_2$	$^2H_3$	$^2H_0$	$^2H_1$	$^2H_2$	$^2H_3$	$^2H_0$	$^2H_1$	$^2H_3$
control	30.9	20.3	46.1	2.6	24.3	28.7	45.9	1.1	55.6[a]	29.9	14.5
	(100)	(82.9)	(165.8)	(37.7)	(100)	(126.6)	(201.8)	(24.2)	(100)	(60.1)	(29.4)
									56.0[b]	29.7	14.3
									(100)	(58.0)	(28.2)
									56.2[c]	30.0	13.8
									(100)	(66.6)	(33.7)
2 mM Guo	39.4	19.9	38.2	2.5	29.8	30.2	38.1	1.9	70.3[a]	20.5	9.2
	(100)	(67.6)	(110.6)	(25.5)	(100)	(110.2)	(139.4)	(20.2)	(100)	(35.4)	(14.8)
									68.4[b]	21.7	9.9
									(100)	(36.7)	(16.0)
									72.6[c]	17.9	9.5
									(100)	(38.0)	(18.2)
50 mM B_{12}	30.4	18.5	46.6	4.5	25.1	28.7	45.8	0.4	55.8[a]	31.5	12.7
	(100)	(78.0)	(168.8)	(44.2)	(100)	(122.8)	(194.7)	(20.6)	(100)	(62.7)	(26.2)
									52.7[b]	33.0	14.3
									(100)	(67.6)	(30.3)
									55.1[c]	33.0	11.9
									(100)	(73.2)	(31.4)

[a] Determined from the *m/z* 154 ion in the 70-eV spectra of glycine. [b] Determined from the *m/z* 172 ion in the 70-eV spectra of glycine. [c] Determined from the *m/z* 228 (MH^+) ion in the CI spectra of glycine.

Table III: Incorporation of DL-Serine-*3,3-d*$_2$ into Methionine and Thymine by *E. coli* Mutants

	distribution of ^2H in Met *m/z* 61 ion				distribution of ^2H in Thy *m/z* 126 (M^+) ion			
strain/mutant	$^2H_0$	$^2H_1$	$^2H_2$	$^2H_3$	$^2H_0$	$^2H_1$	$^2H_2$	$^2H_3$
E. coli B WG 1143 *serA*[a]	36.7	7.4	54.2	1.7	26.1	8.1	63.5	2.3
	(100)	(37.3)	(156.3)	(31.1)	(100)	(39.5)	(245.6)	(30.9)
E. coli B WG 1143 *serA*[a]	30.7	9.6	58.6	1.1	34.2	7.2	56.5	2.1
grown with 2 mM Guo	(100)	(48.5)	(201.6)	(38.3)	(100)	(29.6)	(169.8)	(21)
E. coli K12	34.7	7.5	57.0	0.8	26.2	7.4	65.5	0.9
CU 331 *serA*[a,b]	(100)	(38.8)	(173.2)	(31.8)	(100)	(37.0)	(254.5)	(25.8)

[a] Deuterium was not present in the glycine isolated from these cells. [b] Serine isolated from these cells had 25.3% $^2H_0$ and 74.7% $^2H_2$. No monodeuterated serine was detected.

methionine. Cells grown with serine-*2,3,3-d*$_3$ contained 18–30% monodeuterated methyl groups in the methionine and thymine (Table II) whereas cells grown with serine-*3,3-d*$_2$ contained only 7–8% monodeuterated methyl groups (Table III). This clearly indicates that an appreciable fraction of these methyl groups, at least 15–20% of the total, are derived from the C-2 of serine. That the incorporation is proceeding via glycine is confirmed by the presence of deuterated glycine when serine-*2,3,3-d*$_3$ is fed (Tables I and II) and by the absence of deuterated glycine when serine-*3,3-d*$_2$ is fed (data not shown).

The actual incorporation of the C-2 of glycine into the methyl groups, however, will be much higher than these numbers indicate since many of the glycine C-2 deuteriums are lost at some unknown stage in its metabolism. This loss of deuterium has been demonstrated in every sample tested (Tables I and II) and cannot be accounted for by chemical exchange during hydrolysis and derivative formation. I have no explanation for the occurrence of glycine with two deuteriums, but it is clear that they do not come from a reverse of the glycine cleavage reaction since the glycine from cells grown with serine-*3,3-d*$_2$ has no label.

Exchange of Serine C-3 Protons. The methyl groups of methionine and thymine still contain about 8% monodeuterated species when serine-*3,3-d*$_2$ is fed, indicating that exchange of the C-3 serine protons does occur. This exchange could occur by the enzymatic interconversion of 5,10-methylene-H_4folate to either 5,10-methenyl-H_4folate or 5-methyl-H_4folate. Either of these reactions functioning under reversible conditions would result in deuterium loss. In the 5,10-methylene-H_4folate reductase reaction (Katzen & Buchanan, 1965), this would result from the formation of monodeuterated 5,10-methylene-H_4folate from dideuterated 5-methyl-H_4folate. Methylenes with no deuterium would be produced, to a small extent, only after two passes through the reaction.

The 5,10-methylene-H_4folate dehydrogenase reaction, on the other hand, would catalyze the loss of one deuterium from 5,10-methylene-H_4folate upon its conversion into 5,10-methenyl-H_4folate. This would involve removal of one deuterium from the methylene group and its transfer to the A side of $NADP^+$ (Ramasastri & Blakley, 1964). The resulting NADPD would equilibrate with unlabeled NADPH in the cell, and the reverse reaction would produce 5,10-methylene-H_4-folate containing one deuterium. However, in a second pass through the reaction, assuming the $NADP^+$ oxidation to be stereospecific, we would still obtain methylene groups with one deuterium. This exchange mechanism would, therefore, produce methyl groups with only one deuterium (assuming a metabolic sink for the deuterium in the labeled NADPH). However, the dehydrogenase reaction is not stereospecific for the methylene protons with a loss of about 50% stereochemical purity occurring with each pass of the labeled 5,10-methylene-H_4folate through the reaction (Tatum et al., 1977). Thus, this reaction could also account for the complete loss of deuterium from some of the methylene groups of 5,10-methylene-H_4folate by multiple passes of the reactants and products through the reaction.

The dehydrogenase reaction would also be implicated in the exchange of the protons due to its ready reversibility and an equilibrium constant of 0.14 at pH 6.9, which is close to unity (Uyeda & Rabinowitz, 1967). By comparison to other known dehydrogenases, it could be argued that the reaction proceeds intracellularly under equilibrium conditions (Krebs & Veech,

1969). That the exchange is in fact occurring from 5,10-methylene-H_4folate was confirmed when the thymine, which also arises from 5,10-methylene-H_4folate, showed molecules with one deuterium after isolation from the cells (Tables II and III).

In order to test the involvement of 5,10-methylene-H_4folate dehydrogenase in the exchange reaction, it was necessary to grow *E. coli* cells in the presence of guanosine, which has been shown to repress the synthesis of this enzyme by about 40% (Taylor et al., 1966). With guanosine present in the medium, not only would synthesis of the enzyme be repressed but also, more importantly, the flux of C_1 units through the folate pathway would be greatly reduced since 10-formyl-H_4folate would not be used for purine biosynthesis (Newman & Magasanik, 1963). This would increase the chances that the product of the reaction, 5,10-methenyl-H_4folate, would be converted back to 5,10-methylene-H_4folate, which would result in increased deuterium loss and the production of both methionine and thymine with a shift in distribution from molecules containing two deuteriums to those containing either none or one. This is consistent with the data in Tables II and III where an increase in the number of molecules containing one deuterium over those with two deuteriums can be seen for both methionine and thymine on the addition of guanosine.

Similarly, growth with B_{12} is known to strongly repress the synthesis of the reductase (Katzen & Buchanan, 1965; Milner et al., 1969; Kung et al., 1972). Since the addition of B_{12} is not expected to change the flux of C_1 units going to methionine, and since the same amount of methionine is needed with or without added B_{12}, we would expect less exchange with added B_{12} if the reductase is involved in the exchange. Table II shows that B_{12} has no effect on the deuterium distribution, which indicates that the reductase is not likely to be involved in the deuterium exchange.

The simplest explanation for the difference observed between the deuterium incorporated into the thymine methyl and that incorporated into the methionine methyl is that there are at least two separate sources of 5,10-methylene-H_4folate in the cell. One of these sources is preferentially labeled during the glycine cleavage reaction and the other is preferentially labeled during the serine hydroxymethyltransferase reaction. These two sources of 5,10-methylene-H_4folate, with a finite degree of selectivity, are then used in the biosynthesis of the thymine and methionine methyl groups. The chemical basis for these two sources of the same biosynthetic unit may result from the presence of folate polyglutamates present in the cells.

The occurrence of folate polyglutamates in bacteria is well documented (Salem et al., 1972; Baugh et al., 1974; Powers & Snell, 1976), and folate polyglutamates have been shown to be differentially utilized in several folate-dependent reactions (Baggott & Krumdieck, 1979). These include the thymidylate synthetase reaction (Kisliuk et al., 1974), the ketopantoate hydroxymethyltransferase reaction (Powers & Snell, 1976), the biosynthesis of methionine (Foster et al., 1964; Guest et al., 1962, 1964; Guest & Woods, 1965; Coward et al., 1975), and the dihydrofolate reductase reaction (Coward et al., 1974).

Whether this is true for the glycine cleavage reaction and/or the serine hydroxymethyltransferase reaction is, at present, not known although it seems very likely that these reactions would show some selectivity in their reaction with the different folate polyglutamates. It should be pointed out, however, that only one of these reactions leading to the production of 5,10-methylene-H_4folate polyglutamates needs to be selective in order to generate the differences in the deuterium distribution observed in the methyl groups.

Another explanation for the increased occurrence of thymine containing an excess of one deuterium over that observed in the methionine would be the incorporation of an additional deuterium into the methyl group from the A side of NADPH during the thymidylate synthetase reaction (Blakley et al., 1963; Pastore & Friedkin, 1962). The NADPH would, in turn, be labeled with deuterium during the 5,10-methylene-H_4folate dehydrogenase reaction. The absence of this reaction is confirmed by comparing the observed isotopic patterns seen in the methionine and thymine methyl groups. If an additional portion of a single deuterium population were added to that observed in the methionine methyl, we would see thymine molecules with three deuteriums, which, as shown in Table II, are not present. In addition, it is impossible to add an additional fractional part of one deuterium to the methionine distribution to generate the thymine distribution.

Acknowledgments

I thank Kim Harich for his assistance in obtaining the mass spectral data and Linda White for editorial help in the preparation of the manuscript.

Registry No. Serine, 56-45-1; methionine, 63-68-3; thymine, 65-71-4; glycine, 56-40-6; 5,10-methylene-H_4folate dehydrogenase, 9029-14-5.

References

Baggott, J. E., & Krumdieck, C. L. (1979) in *Chemistry and Biology of Pteridines* (Kisliuk, R. L., & Brown, G. M., Eds.) pp 347–351, Elsevier/North-Holland, New York.

Baugh, C. M., Braverman, E., & Nair, M. G. (1974) *Biochemistry 13*, 4952–4957.

Biemann, K. (1962) *Mass Spectrometry*, McGraw-Hill, New York.

Blakley, R. L. (1969) *The Biochemistry of Folic Acid and Related Pteridines*, North-Holland, Amsterdam.

Blakley, R. L., Ramasastri, B. V., & McDougall, B. M. (1963) *J. Biol. Chem. 238*, 3075–3079.

Cathou, R. E., & Buchanan, J. M. (1963) *J. Biol. Chem. 238*, 1746–1751.

Cheeseman, P., & Crosbie, G. W. (1966) *Biochem. J. 99*, 24P.

Coward, J. K., Parameswaran, K. N., Cashmore, A. R., & Bertino, J. R. (1974) *Biochemistry 13*, 3899–3903.

Coward, J. K., Chello, P. L., Cashmore, A. R., Parameswaran, K. N., DeAngelis, L. M., & Bertino, J. R. (1975) *Biochemistry 14*, 1548–1552.

Crosbie, G. W. (1966) *Biochem. J. 99*, 21P–22P.

Dev, I. K., & Harvey, R. J. (1982) *J. Biol. Chem. 257*, 1980–1986.

Foster, M. A., Tejerina, G., Guest, J. R., & Woods, D. D. (1964) *Biochem. J. 92*, 476–488.

Gelpi, E., Koenig, W. A., Gilbert, J., & Oro', J. (1969) *J. Chromatogr. Sci. 7*, 604–613.

Guest, J. R., & Woods, D. D. (1965) *Biochem. J. 97*, 500–512.

Guest, J. R., Friedman, S., & Foster, M. A. (1962) *Biochem. J. 84*, 93P–94P.

Guest, J. R., Friedman, S., Foster, M. A., Tejerina, G., & Woods, D. D. (1964) *Biochem. J. 92*, 497–504.

Harvey, R. J., & Dev, I. K. (1975) *Adv. Enzyme Regul. 13*, 99–124.

Hogness, D. S., Cohn, M., & Monod, J. (1955) *Biochim. Biophys. Acta 16*, 99–116.

Jordan, P. M., & Akhtar, M. (1970) *Biochem. J. 116*, 277–286.

Katzen, H. M., & Buchanan, J. M. (1965) *J. Biol. Chem. 240*, 825–835.

Kisliuk, R. L. (1963) *J. Biol. Chem. 238*, 397–400.

Kisliuk, R. L., Gaumont, Y., & Baugh, C. M. (1974) *J. Biol. Chem. 249*, 4100–4103.
Koch, A. L., & Levy, H. R. (1955) *J. Biol. Chem. 217*, 947–957.
Kochi, H., & Kikuchi, G. (1974) *J. Biochem. (Tokyo) 75*, 1113–1127.
Krebs, H. A., & Veech, R. L. (1969) *FEBS-Symp. No. 17*, 101–109.
Kung, H., Spears, C., Greene, R. C., & Weissbach, H. (1972) *Arch. Biochem. Biophys. 150*, 23–31.
Leimer, K. R., Rice, R. H., & Gehrke, C. W. (1977) *J. Chromatogr. 141*, 121–144.
Lomax, M. S., & Greenberg, G. R. (1967) *J. Biol. Chem. 242*, 1302–1306.
Marshak, A., & Vogel, H. J. (1951) *J. Biol. Chem. 189*, 597–605.
Meedel, T. H., & Pizer, L. I. (1974) *J. Bacteriol. 118*, 905–910.
Milner, L., Whitfield, C., & Weissbach, H. (1969) *Arch. Biochem. Biophys. 133*, 413–419.
Newman, E. B., & Magasanik, B. (1963) *Biochim. Biophys. Acta 78*, 437–448.
Pastore, E. J., & Friedkin, M. (1962) *J. Biol. Chem. 237*, 3802–3810.
Pizer, L. I. (1965) *J. Bacteriol. 89*, 1145–1150.
Powers, S. G., & Snell, E. E. (1976) *J. Biol. Chem. 251*, 3786–3793.
Ramasastri, B. V., & Blakley, R. L. (1964) *J. Biol. Chem. 239*, 112–114.
Rotman, B., & Spiegelman, S. (1954) *J. Bacteriol. 68*, 419–429.
Salem, A. R., Pattison, J. R., & Foster, M. A. (1972) *Biochem. J. 126*, 993–1004.
Tatum, C. M., Benkovic, P. A., Benkovic, S. J., Potts, R., Schleicher, E., & Floss, H. G. (1977) *Biochemistry 16*, 1093–1102.
Taylor, R. T., Dickerman, H., & Weissbach, H. (1966) *Arch. Biochem. Biophys. 117*, 405–412.
Uyeda, K., & Rabinowitz, J. C. (1967) *J. Biol. Chem. 242*, 4378–4385.
Wall, J. S. (1953) *Anal. Chem. 25*, 950–953.
White, R. H. (1981) *Anal. Biochem. 114*, 349–354.

Cryokinetic Studies of the Intermediates in the Mechanism of Carboxypeptidase A†

Alphonse Galdes, David S. Auld, and Bert L. Vallee*

ABSTRACT: The detection and definition of intermediates in reaction pathways, a problem central to delineation of structure–function relationships in enzymology, have in general resisted solution. We have developed an approach capable of wide application to such studies employing a combination of cryokinetics and cryospectroscopy as exemplified here by using carboxypeptidase A. These studies are performed with a low-temperature stopped-flow instrument which also serves as a cryospectrometer [Auld, D. S. (1979) *Methods Enzymol. 61*, 318]. The intermediates are monitored directly through fluorescence generated by radiationless energy transfer (RET) between enzyme tryptophans and the dansyl group of enzyme-bound substrate. N-Dansylated oligopeptides and their ester analogues exhibit Michaelis–Menten kinetics over the temperature range −20 to +20 °C with k_{cat}/K_m values of (0.3–3) × 10^7 M^{-1} s^{-1} at +20 °C, pH 7.5, and the cryosolvent, aqueous 4.5 M NaCl, does not alter catalysis. There is no chemical evidence of a covalent acyl enzyme intermediate for any of the peptide or ester substrates studied. However, concurrent cryospectroscopy shows that peptides and esters form metallo intermediates whose spectra differ strikingly from one another and from that of the enzyme alone [Geoghegan, K. F., Galdes, A., Martinelli, R. A., Holmquist, B., Auld, D. S., & Vallee, B. L. (1983) *Biochemistry* (in press)]. The cryokinetic data demonstrate for the first time the existence of *two* intermediates during the hydrolysis of both peptides and esters. At −20 °C, the formation, interconversion and breakdown of these intermediates results in three distinct fluorescence steps during substrate hydrolysis: (1) a rapid increase in signal intensity reflects the formation of the Michaelis complex, ES_1, in <15 ms; (2) a slower exponential increase in signal intensity signifies formation of a second hitherto unknown intermediate, ES_2; (3) a slow decrease in signal intensity reflects separation of the dansyl product from the enzyme. All rate and equilibrium constants for the reaction scheme $E + S \rightleftharpoons ES_1 \rightleftharpoons ES_2 \rightarrow E + P$ have been determined. The reversible interconversion of ES_1 and ES_2 shows that the C-terminal product is not liberated prior to the rate-limiting step and, hence, deacylation cannot be rate limiting. The cryokinetic studies in conjunction with the chemical evidence demonstrate that there is no acyl intermediate in either ester or peptide hydrolysis. The present cryokinetics and the concurrent cryospectroscopy show that peptides and esters form different metallo intermediates and that these two types of substrates are hydrolyzed through different mechanisms.

Radiationless energy transfer (RET)[1] allows direct detection of transient enzyme–substrate (ES) complexes, thereby providing a powerful approach to the investigation of enzymatic reactions (Auld, 1977; Lobb & Auld, 1979, 1980). RET arises from the transfer of energy between a fluorescent acceptor–donor pair, e.g., enzyme tryptophanyl residues and the blocking

† From the Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School, Brigham and Women's Hospital, Boston, Massachusetts 02115. *Received November 12, 1982.* This work was supported in part by Grant-in-Aid GM-24967 from the National Institutes of Health of the Department of Health, Education and Welfare to Harvard Medical School.

[1] Abbreviations: RET, radiationless energy transfer; Dns, dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; OPhe, L-β-phenyllactate; SPhe, α-mercapto-β-phenylpropionate; HPLC, high-performance liquid chromatography; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

0006-2960/83/0422-1888$01.50/0