Providing One-Carbon Units for Biological Methylations: Mechanistic Studies on Serine Hydroxymethyltransferase, Methylenetetrahydrofolate Reductase, and Methyltetrahydrofolate-Homocysteine Methyltransferase

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I. Introduction

This review will stress recent advances in our understanding of the mechanisms of three enzymes that catalyze sequential reactions in the de novo biosynthesis of methyl groups. Serine hydroxymethyltransferase (E.C. 2.1.2.1) is a pyridoxal phosphate-dependent enzyme that catalyzes the physiological reaction shown in eq 1. Methylenetetrahydrofolate reductase (E.C. 1.1.1.68) is a flavoprotein that reduces CH_2-H_4folate[1] (**1**) to CH_3-H_4folate (**2**), as shown in eq 2. The R group shown in eq 2 is para-substituted benzoyl-L-glutamate. Methione synthase (E.C. 2.1.1.13) is a cobalamin-dependent enzyme that transfers the methyl group of CH_3-H_4folate to homocysteine to form methionine, as shown in eq 3.

The activity of these three enzymes is essential for the biosynthesis of methionine in prokaryotic organisms, and in mammals it is required for the regeneration of the methyl group of methionine in support of

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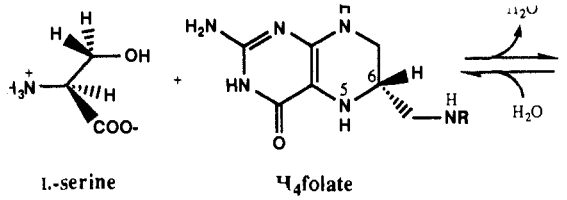
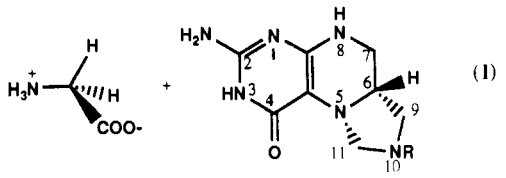
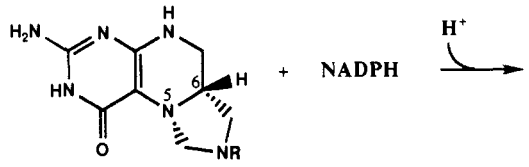


L-serine H_4folate

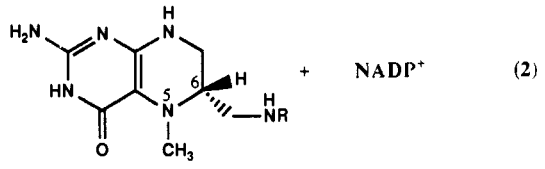


(1)

glycine CH_2-H_4folate (1)



CH_2-H_4folate (1)



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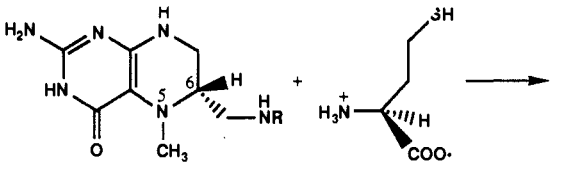
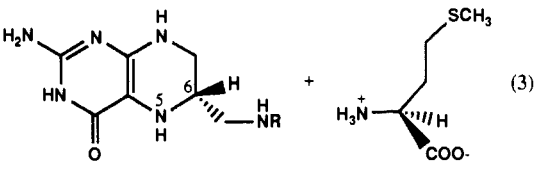
CH_3-H_4folate (2)



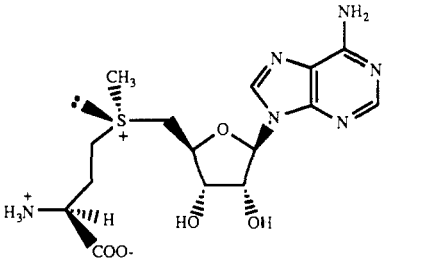
CH_3-H_4folate (2) L-homocysteine



(3)

H_4folate L-methionine

AdoMet-dependent biological methylations. Methionine is an essential amino acid for mammals, since homocysteine is not synthesized, but the methyl group can be supplied by this pathway to remethylate homocysteine formed as a result of methylation reactions that convert AdoMet (3) to AdoHCy followed by hydrolysis of AdoHCy to form adenosine and homocysteine. Serine hydroxymethyltransferase activity provides the major source of one carbon units and glycine for the de novo biosynthesis of purines and of one carbon units for the methylation of dUMP to form dTMP. Due to its important role in support of the synthesis of nucleotides, serine hydroxymethyltransferase is a potential target for chemotherapeutic intervention against cancer, although no clinically effective inhibitors of this enzyme are currently available. Methionine synthase is also a potential target, because inhibition of this enzyme is expected to inhibit nucleotide biosynthesis in cells. The major circulating form of folic acid is CH_3-H_4folate, present as the monoglutamate form. Once this compound enters cells, its conversion to derivatives needed for nucleotide biosynthesis requires the action of methionine synthase.[2] Blockade of methionine synthase activity would be expected to selectively inhibit rapidly dividing cells, because provision of methionine in the diet will meet the needs of nondividing cells for AdoMet and methionine, but not those of dividing cells for folate.



S-adenosyl-L-methionine (3)

Not only do these three enzymes catalyze sequential steps in the metabolic sequence for the de novo biosynthesis of methyl groups, but also these three enzymes share a requirement for the activation of an sp^3 substituent bound to N^5 of a tetrahydropteridine ring system. Methionine synthase must activate the methyl group of CH_3-H_4folate for nucleophilic displacement by cob(I)alamin. Methylenetetrahydrofolate reductase catalyzes the interconversion of CH_2-H_4folate and CH_3-H_4folate and must activate a hydrogen of the methyl group for removal in the oxidation of methyltetrahydrofolate. Serine hydroxymethyltransferase must activate the sp^3 methylene group of CH_2-H_4folate for transfer of formaldehyde or its equivalent to the glycine–aldimine carbanion. In each case, this step or these steps constitute the least well understood aspect of catalysis. It is clear that the redox properties of tetrahydropteridine may facilitate activation of a saturated carbon substituent. Removal of one or more electrons from N^5 would be expected to increase the susceptibility of the substituent to either nucleophilic attack or removal of a proton. Conversely, hydride abstraction from an sp^3 substituent on N^5 may be facilitated by the availability of an electron pair on the attached nitrogen. On the other hand, we need to consider the possibility of a direct nucleophilic attack on the α-carbon of a tertiary amine, a reaction that has evoked little sympathy heretofore from organic chemists. Finally, at least in the case of serine hydroxymethyltransferase, restricted rotation of formaldehyde generated at the active site of the enzyme by retroaldol cleavage may be invoked to avoid either oxidative activation of the tetrahydropteridine or nucleophilic attack on a substituent at N^5. It is hoped that this review will encourage further studies of these interesting and related problems. The literature search was concluded in November 1989.

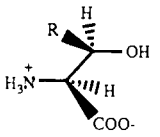
II. Serine Hydroxymethyltransferase

Serine hydroxymethyltransferase has been extensively studied, and several excellent reviews[3] are available. We will briefly summarize the salient features of earlier work, will present newer findings in some

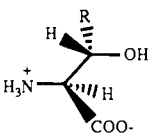
detail, and then will focus on the mechanistic questions that remain to be addressed.

A. Reactions Catalyzed and Their Stereospecificity

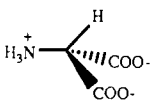
Serine hydroxymethyltransferase catalyzes the aldol cleavage of a number of β-hydroxyamino acids such as L-threonine (4), L-allothreonine (5), and L-*threo*- and L-*erythro*-β-phenylserine (6, 7) and the decarboxylation of aminomalonate (8), in addition to catalyzing the



R = CH_3, L-threonine (4)
R = phenyl, L-threo-β-phenylserine (6)

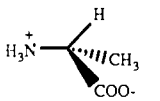


R = CH_3, L-allothreonine (5)
R = phenyl, L-erythro-β-phenylserine (7)



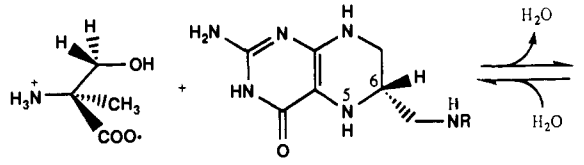
aminomalonate (8)

transfer of the β-carbon of serine to H_4folate. These reactions are summarized in Table I. Only those reactions that generate formaldehyde by the aldol cleavage require the participation of H_4folate, and a central mechanistic question is whether the mechanisms of these H_4folate-dependent reactions proceed by a different route or whether H_4folate is simply required to shuttle formaldehyde in and out of the enzyme's active site. Serine hydroxymethyltransferase also catalyzes rapid exchange of the α-hydrogen of D-alanine in the presence of H_4folate and slowly transaminates D-alanine (9) in the absence of H_4folate.[4] These observations led

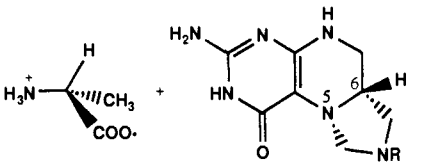


D-alanine (9)

Dunathan[5] to propose that serine hydroxymethyltransferase would labilize the *pro-S* hydrogen of glycine, which corresponds sterically to the α-hydrogen of D-alanine. This stereochemical prediction was confirmed by Besmer and Arigoni[6] and by Jordan and Akhtar,[7] who showed that the interconversion of glycine and serine catalyzed by serine hydroxymethyltransferase occurred with retention of configuration at the α-carbon and resulted in stereospecific loss of the *pro-S* hydrogen of glycine. In the presence of H_4folate, exchange of the *pro-S* hydrogen of glycine is greatly accelerated, but the loss of tritium from residual glycine is reduced if both formaldehyde and H_4folate are present.[7] The hydrogen at the *pro-R* position of glycine is exchanged at least 10-fold more slowly during conversion of glycine and CH_2-H_4folate to serine and H_4folate,[6,7] and in fact α-methylserine (10) can be enzymatically converted to D-alanine in a reaction that requires H_4folate as shown in eq 4.[8] We estimate a value for k_{cat} of 0.4 s^{-1} for this



α-methyl-L-serine (10) H_4folate

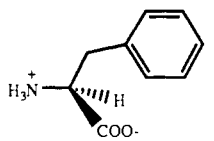


(4)

D-alanine (9) CH_2-H_4folate

reaction (but there is some uncertainty in this number as indicated in footnote *d* to Table I), which is to be compared with a k_{cat} value of 8–20 s^{-1} for the reaction with L-serine. Taken at face value, this observation precludes mechanisms involving β-elimination. However, a direct demonstration that the oxygen of the hydroxymethyl group of serine did not exchange during incubation of the enzyme-bound serine aldimine with nonreactive folate analogues like CH_3-H_4folate would provide stronger evidence against β-elimination. Thus further experiments may be required to determine whether the physiological reaction, viz. the H_4folate-dependent conversion of serine to glycine, proceeds by a mechanism involving β-elimination.

In contrast to the apparent stereospecificity of exchange of the α-hydrogens of glycine, serine hydroxymethyltransferase catalyzes slow exchange of the α-hydrogen of L-phenylalanine (11), which corresponds

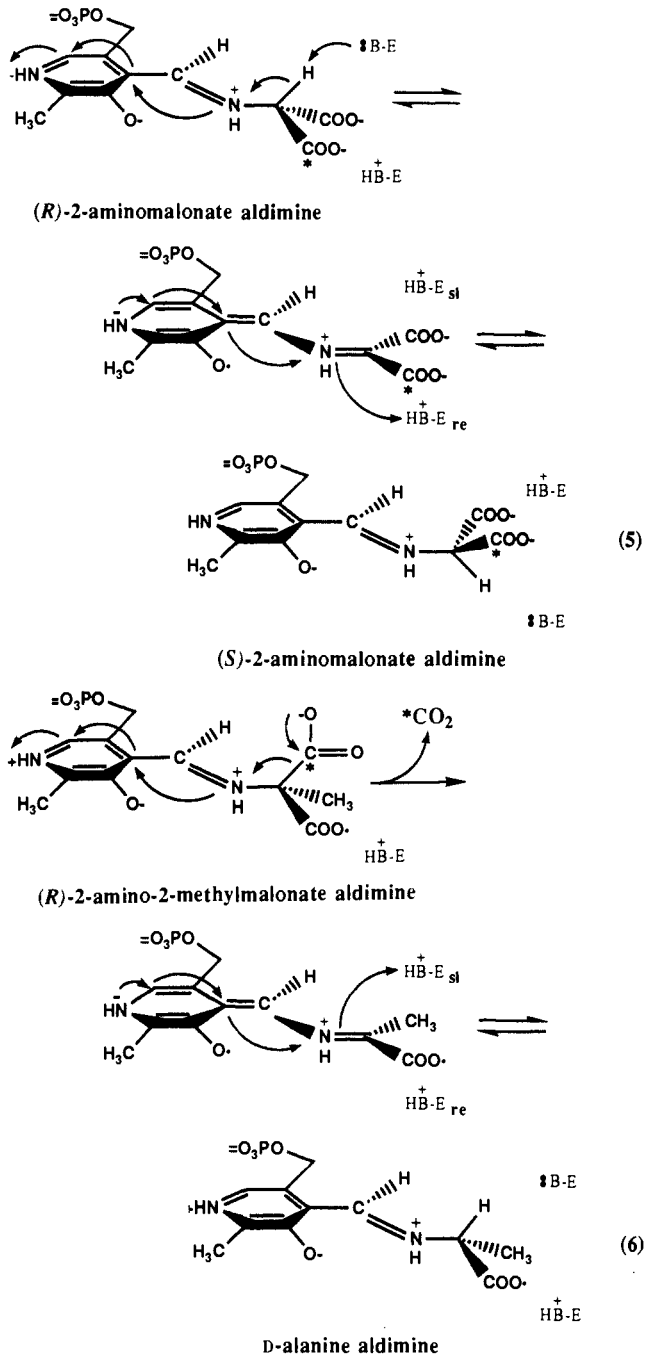


L-phenylalanine (11)

to the *pro-R* hydrogen of glycine, and the external aldimine formed between pyridoxal phosphate and L-phenylalanine is slowly converted to a quinoid species with absorbance at 503 nm.[9] Quinoid species with similar absorbance properties are seen when serine hydroxymethyltransferase is incubated with glycine or D-alanine, and the concentration of the quinoid species is greatly increased in the presence of H_4folate or folate analogues.[10] Quinoid species are also seen during turnover of serine hydroxymethyltransferase with β-phenylserine substrates.[11] Formation of a quinoid species is associated with exchange of the α-hydrogen of glycine, D-alanine, and L-phenylalanine, and H_4folate accelerates the rate of this exchange. On the basis of these observations, the quinoid species are thought to represent resonance-stabilized carbanions formed by loss of the α-hydrogen from the external aldimine. The presumed structure of the quinoid aldimine (12) derived from glycine is shown in Scheme I.

Serine hydroxymethyltransferase catalyzes slow racemization of D-alanine[12] and rapid racemization of aminomalonate when the carboxylates are isotopically

distinguished.[13] In eqs 5 and 6 the *R* carboxylate indicated with an asterisk is labeled with ^{13}C so it may be distinguished experimentally from the unlabeled *S* carboxylate. As shown in eq 5, racemization implies protonation of the aldimine face opposite that of initial proton abstraction, a result consistent with the presence of an enzyme-bound base adjacent to each face of the aldimine capable of donating or abstracting a proton.[12] With the possible exception of aminomalonate, abstraction and return of the *S* or *pro-S* hydrogen is clearly the kinetically favored process. The presence of a second carboxylate would be predicted to increase greatly the thermodynamic acidity of the aminomalonate aldimine proton and increase the facility of exchange from either face. This rapid racemization occurs prior to decarboxylation and explains the previous observation of nonstereospecific decarboxylation of aminomalonate.[14] However, the stereoselective decarboxylation of the *pro-R* carboxylate of 2-amino-2-methylmalonate[13] leaves behind an enzyme-bound quinoid identical with that formed by the deprotonation of D-alanine, and this species is protonated with overall retention from the *si* face, as shown in eq 6. The presence of the α-methyl group precludes the rapid racemization seen prior to decarboxylation in the parent aminomalonate, and the stereoselective decarboxylation implies that the enzyme recognizes one orientation of the α-methyl group with respect to the plane of the aldimine.



(5)

(6)

The stereochemical specificity at the β-carbon of L-amino acids is relaxed. Thus, the enzyme mediates aldol cleavage of both L-threonine and L-allothreonine[15] and of *threo*-β-phenylserine and *erythro*-β-phenylserine,[11] although L-allothreonine and *erythro*-β-phenylserine are the better substrates (Table I) with both higher k_{cat} values and lower K_m values.

B. Stereochemical Analysis of the Physiological Reaction

In the transfer of the β-carbon of serine to H_4folate, (3*R*)-[3-^{3}H]serine is converted to (11*R*)-[11-^{3}H]CH_2-H_4folate.[16] Initial studies with enzyme from rat liver slices[16a] or with purified enzyme from rabbit muscle[16b] suggested that the transfer was associated with loss of about 50% of the stereochemical purity of the transferred carbon center, and this racemization was thought to indicate the formation of a "free formaldehyde" intermediate in the catalysis of the reaction shown in eq 1.[16b] The analysis of the stereochemistry of the product CH_2-H_4folate generated from purified rabbit liver enzyme was accomplished by coupling the reaction with an excess of methylenetetrahydrofolate dehydrogenase obtained from yeast and analyzing the resulting products, NADPH and CH^+=H_4folate, for their tritium content. More recent investigations, with purified serine hydroxymethyltransferase and methylenetetrahydrofolate dehydrogenase from pig liver, resulted in only ~10% loss of stereochemical purity.[16c] Although the differences observed could be due to the slightly different reaction conditions, or to the different sources of the enzymes, they may reasonably be interpreted as indicating that the loss of stereochemical purity may be an artifact of the experimental procedure, rather than an intrinsic property of the enzyme-catalyzed reaction.[16c] Indeed, the coupled rabbit liver serine hydroxymethyltransferase/thymidylate synthase reaction used by Tatum et al.[17] to synthesize dTMP for stereochemical analysis of the thymidylate synthase reaction resulted in dTMP of unexpectedly high stereochemical purity, with only ~23% racemization. In the original experiments of Biellmann and Schuber,[16a] serine was synthesized by incubating glycine and [^{3}H]formate with rat liver slices and then analyzed by stereospecific conversion to ethanol. The degree of coupling observed in such conditions will depend in part on the degree to which the trifunctional methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase/formyltetrahydrofolate synthetase protein activity is in excess over the activity of serine hydroxymethyltransferase in liver slices. If a sufficient excess of the trifunctional enzyme is present, racemization of CH_2-H_4folate may occur in solution prior to its consumption in the serine hydroxymethyltransferase-catalyzed conversion of glycine to serine.[16b]

TABLE I. Reactions Catalyzed by Serine Hydroxymethyltransferase

enzyme source	conditions: pH, temp (°C)	reaction catalyzed	K_m, mM	k_{cat}, s^{-1}	ref
lamb liver	7.5, 25	D,L-allothreonine → glycine + acetaldehyde	1.3	0.56	*a*
		L-threonine → glycine + acetaldehyde	32	0.09	
		D,L-*erythro*-β-phenylserine → benzaldehyde + glycine	9.5	21	
		D,L-*threo*-β-phenylserine → benzaldehyde + glycine	84	7	
		D,L-*erythro*-β-phenylserine methyl ester → benzaldehyde + glycine methyl ester	70	29	
rat liver	7.5, 37	L-serine + H_4folate → glycine + CH_2-H_4folate	0.45	18.9	13
	6.0, 37	aminomalonate → glycine + CO_2	12	58.7	
	6.3, 37	L-allothreonine → glycine + acetaldehyde	1	19.6	
rabbit liver (cytosolic)	7.3, 30	L-serine + H_4folate → glycine + CH_2-H_4folate	1.3	8.3	*b*
	7.2, 37	L-serine → glycine + formaldehyde	0.7	4.7×10^{-5}	20
		α-methyl-L-serine + H_4folate → D-alanine + CH_2-H_4folate		0.4[d]	8
		exchange of *pro-S* α-hydrogen of glycine		0.020	*c*
		exchange of *pro-S* α-hydrogen of glycine (+H_4folate)		2	
		glycine + CH_2-H_4folate → L-serine + H_4folate		1.3	
		glycine + formaldehyde → L-serine		0.008	
		acetaldehyde + glycine → L-threonine		0.16	

[a] Reference 9. [b] Schirch, V.; Hopkins, S.; Villar, E.; Angelaccio, S. *J. Bacteriol.* **1985**, *163*, 1. [c] Chen, H. S.; Schirch, L. *J. Biol. Chem.* **1973**, *248*, 3631. [d] In ref 8, the time of incubation of the assays in which α-methylserine was converted to D-alanine is not given. We have assumed an incubation time of 10 min. The value of k_{cat} so obtained is consistent with the statement in ref 8 that 10–15× more enzyme was required for assays with α-methylserine than with serine.

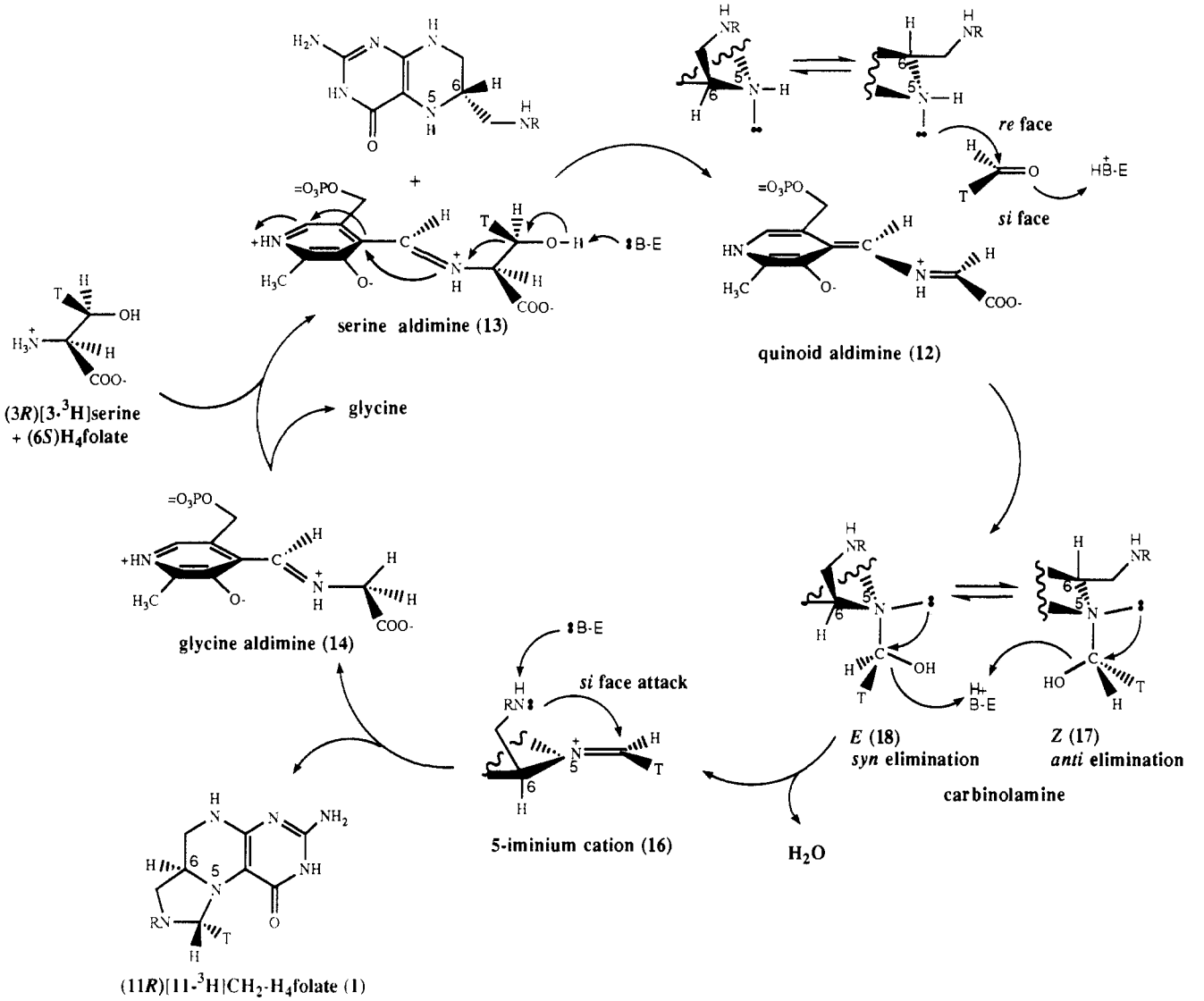
C. Possible Reaction Mechanisms for the Physiological Reaction

The observation of a high degree of stereospecificity in the serine hydroxymethyltransferase reaction necessitates a review of possible mechanisms for the conversion of serine and H_4folate to glycine and CH_2-H_4folate. The mechanism originally proposed by Jordan and Akhtar,[7] and strongly supported by the observation of substantial racemization of the transferred carbon, is shown in Scheme I. This mechanism is attractive because it involves an aldol cleavage, entirely analogous to the aldol cleavages seen with other β-hydroxyamino acid substrates. The role of H_4folate, according to this mechanism, is to facilitate the transport of formaldehyde in and out of the active site of the enzyme. The mechanism for the condensation of formaldehyde with H_4folate follows that proposed for the nonenzymatic reaction.[18] At neutral pH, the rate-limiting step for the nonenzymatic reaction is dehydration of the N^5-carbinolamine. From the data of Kallen and Jencks,[18] we estimate a rate constant of about 2 s^{-1} for this dehydration at pH 7.

There are a number of stereochemical constraints imposed on this reaction mechanism by the experimental data, many of which were not known at the time of the original proposal. A retroaldol cleavage mechanism consistent with these constraints is presented in Scheme I. The proposal must be consistent with the observation that the methylene group of serine is transferred to H_4folate with a high degree of stereospecificity. The first conclusion to be drawn is that the liberated formaldehyde must not possess rotational freedom, since rotation would present both faces of formaldehyde derived from chiral serine to the quinoid nucleophile and lead to racemization of the methylene group. It follows either that H_4folate must be positioned to accept formaldehyde as it is liberated at the active site or that the active site is able to prevent rotation of the formaldehyde unit that is in equilibrium with its serine aldimine precursor. Protonation of the quinoid aldimine (12) and retroaldol cleavage of the serine aldimine (13) are known to occur at the *si* face of the imine,[19] and the stereochemical results require that N^5 of H_4folate attacks only the *re* face of the formaldehyde. These constraints limit the possible orientations of the three players in the reaction and suggest that formaldehyde, if formed, need not diffuse far to either acceptor, since diffusion would likely be associated with rotation.

A further stereochemical consideration arises once the carbinolamine intermediate has been formed; two possible isomers about the N^5 nitrogen are possible, and these are interconvertable by a nitrogen inversion. In order to obtain CH_2-H_4folate with the observed stereochemistry, cyclization of the 5-iminium cation (16) must occur by attack of N^{10} on the *si* face of the iminium ion. This is turn constrains the possibilities for the dehydration of the carbinolamine. If the elimination of water is antiperiplanar, as proposed for the ring opening of CH_2-H_4folate to give the 5-iminium cation,[20] then the two adjacent alkyl groups (on N^5 and C^6) must adopt the less favored *Z* configuration (17) in order to account for the observed stereochemistry of the eliminated product. The enzyme would have to selectively facilitate both the formation and dehydration of the *Z* isomer. In order for elimination to occur from the *E* isomer (18), which is presumably the energetically favored isomer in aqueous solution, the enzyme would have to catalyze an elimination in which the electron pair on nitrogen and the departing water possess the syn relationship. Viewed in the reverse direction, hydration of the 5-iminium cation must occur on the *si* face of the iminium group, the face shared by the bulk of the aryl portion of the molecule positioned above the *si* face by the stereocenter at C^6. All other examples of nucleophilic attack on H_4folate derivatives for which the stereochemistry is known occur from the opposite, less sterically hindered face.[16c,20] In the forward direction, addition of formaldehyde must also occur at the more sterically hindered face of H_4folate. Finally, one can indirectly support the formation of the 5-iminium cation (16) as an intermediate in the interconversion of CH_2-H_4folate and the carbinolamine. If the sequence were to proceed to the N^5-carbinolamine through a direct, nucleophilic ring opening of CH_2-H_4folate, the

SCHEME I. Mechanism for Serine Hydroxymethyltransferase Involving Retroaldol Cleavage[a]



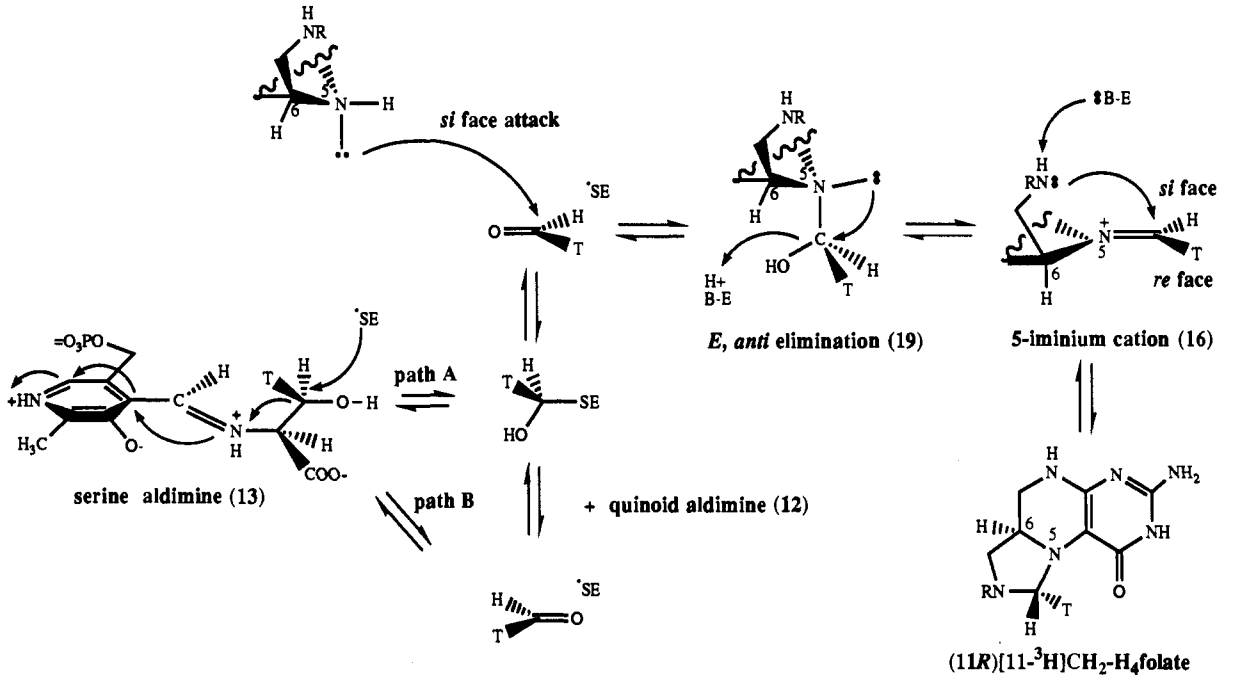
[a] Serine is depicted as forming a Schiff base imine with the pyridoxal phosphate cofactor. Free formaldehyde is liberated by general-base-catalyzed aldol cleavage and trapped by one of two possible isomers of H_4folate, which are related by nitrogen inversion. Rotation of the carbinolamine intermediate about the N^5-carbinolamine carbon bond prepares it for either syn or anti elimination of water, with the constraint that the *si* face of the imine must be presented to N^{10} for ring closure to form CH_2-H_4folate. The apparent violation of Baldwin's rule in the 5-endo-trig closure of the 5-iminium cation to form CH_2-H_4folate has been defended by Slieker and Benkovic.[20] Not shown is the reprotonation of the quinoid aldimine species (see Scheme III).

opposite stereochemistry around the chiral N^5-carbinol would be obtained.

Other concerns about this mechanism arise due to the demonstration that serine hydroxymethyltransferase does not catalyze the condensation of formaldehyde and tetrahydrofolate or the dehydration of the N^5-carbinolamine.[21] Because formaldehyde is hydrated in an aqueous solution, but may be maintained in its dehydrated form at the active site of the enzyme, the failure to observe enzyme-catalyzed condensation of formaldehyde and H_4folate may be explained, but the failure to observe enzyme-catalyzed dehydration of the carbinolamine is a more serious objection. As noted above, the enzyme most probably would have to catalyze interconversion of the *Z* isomer of the carbinolamine with the 5-iminium cation. Taken together, these observations suggest that we ought seriously to consider alternate mechanisms for the transfer of the β-carbon of serine to H_4folate.

An alternative mechanism involving formation of a thiohemiacetal is depicted in Scheme II. Here the serine aldimine (13) is converted to a quinoid aldimine (12) and a thiohemiacetal by either direct nucleophilic displacement (path A) or by sequential retroaldol cleavage and attack of the thiolate on nascent formaldehyde (path B). A carbinolamine with the favored *E* conformation (19) can be formed either from formaldehyde liberated from the thiohemiacetal or by direct nucleophilic attack (not shown), and an anti elimination then gives rise to a 5-iminium cation (16) with the requisite stereochemistry. This intermediate undergoes ring closure to yield CH_2-H_4folate. The most attractive feature of this alternative mechanism is the possibility of anti elimination from the favored *E* conformation of the carbinolamine. However, to avoid the possibility of rotation of nascent formaldehyde, two direct displacements would be required. The first involves the expulsion of the quinoid aldimine, which is a moder-

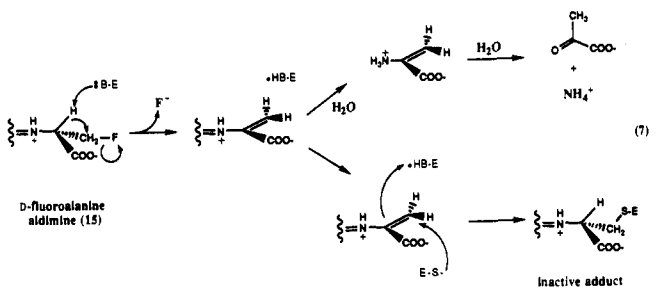
SCHEME II. Mechanism for Serine Hydroxymethyltransferase Involving Thiohemiacetal Formation[a]



[a] An enzyme-bound thiolate residue is depicted as participating in the transfer of the formaldehyde unit, and two mechanistic extremes for the process are presented. Concerted transfer of the carbinol by direct nucleophilic attack of thiolate (path A) achieves the same intermediate thiohemiacetal as a sequence in which free formaldehyde is trapped by a thiolate (path B). Liberation of formaldehyde from the thiohemiacetal presents the *si* face to H_4folate (both quinoid aldimine and H_4folate acceptors now communicate with the *si* face). The stereochemically required intermediate (**16**) can now be obtained by anti elimination from the *E* isomer, and water can reversibly add to the less hindered *re* face of the 5-iminium cation (**16**).

ately good leaving group, while the second requires expulsion of the active center thiolate residue.

An active site thiol has been identified in lamb and rabbit liver serine hydroxymethyltransferase from a study of 3-haloalanine derivatives as suicide substrates for these enzymes.[22] Equation 7 shows the inactivation



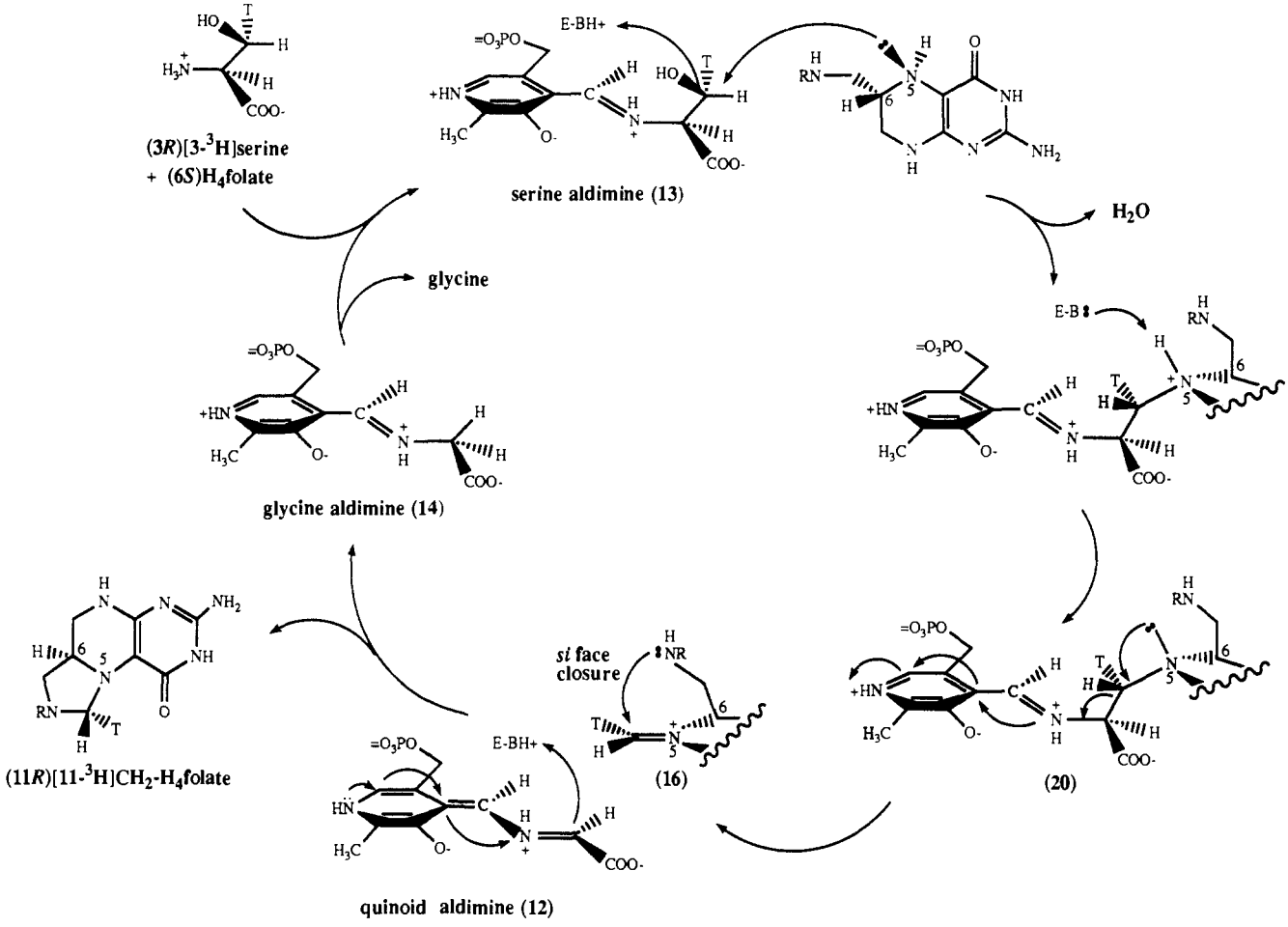
of the enzyme by D-fluoroalanine. Following formation of the aldimine of D-fluoroalanine (**15**), the enzyme abstracts the available α-hydrogen (which corresponds to the *pro-S* hydrogen in glycine) and β-elimination occurs to release fluoride ion. The aminoacrylate aldimine intermediate then partitions between a benign hydrolysis pathway, giving pyruvate and ammonium ion, and a pathway in which the aminoacrylate aldimine serves as a Michael acceptor for an active site thiolate leading to covalent modification. Addition of H_4folate to the enzyme system accelerates the rate of formation of pyruvate and the rate of enzyme inactivation by ~200-fold. This result is consistent with the analogous stimulation of the rate of exchange of the *pro-S* hydrogen of glycine or D-alanine aldimine by H_4folate.[10]

In the absence of added H_4folate, serine hydroxymethyltransferase catalyzes an extremely slow aldol cleavage of serine to form formaldehyde and glycine,[21] and release of formaldehyde from the enzyme appears to be the rate-limiting step in this catalytic reaction. It has been proposed that formaldehyde is retained on the enzyme in an imine or thiohemiacetal linkage,[3b] but direct evidence for this proposal is lacking. The aldol cleavage of threonine and β-phenylserine does not result in replacement of the oxygen of ^{18}O-labeled substrates and precludes mechanisms involving β-elimination with these substrates.[23]

Alternate mechanisms presume that the serine C^{α}–C^{β} bond is labilized by overlap with the π-system of the aldimine and that this activation renders the β-carbon more electropositive. Such a mechanism might involve a nucleophilic attack by N^5 of the H_4folate acceptor on the β-carbon of the serine aldimine to eliminate the quinoid species without the intermediacy of formaldehyde, analogous to the reaction proposed by Scheme II in which an enzyme-bound thiolate is the leaving group. In the reverse direction, the bimolecular displacement of H_4folate in such a model would orient the quinoid nucleophile along the N^5–C bond axis of the carbinolamine in order to maximize overlap with the N^5–C antibonding orbital, but nonbonding electrons on the carbinolamine oxygen are presumably able to overlap as well and offer competition for orbital occupancy. This sequence also suggests that nucleophilic displacement of folate is more facile than carbinolamine deprotonation by the quinoid anion.[24]

Still another alternative mechanism is outlined in Scheme III. In the direction shown, the first step is proposed to be a direct, reversible, nucleophilic attack

SCHEME III. Alternate Mechanism for Serine Hydroxymethyltransferase Requiring Direct Nucleophilic Attack of H_4folate on the Serine Aldimine[a]



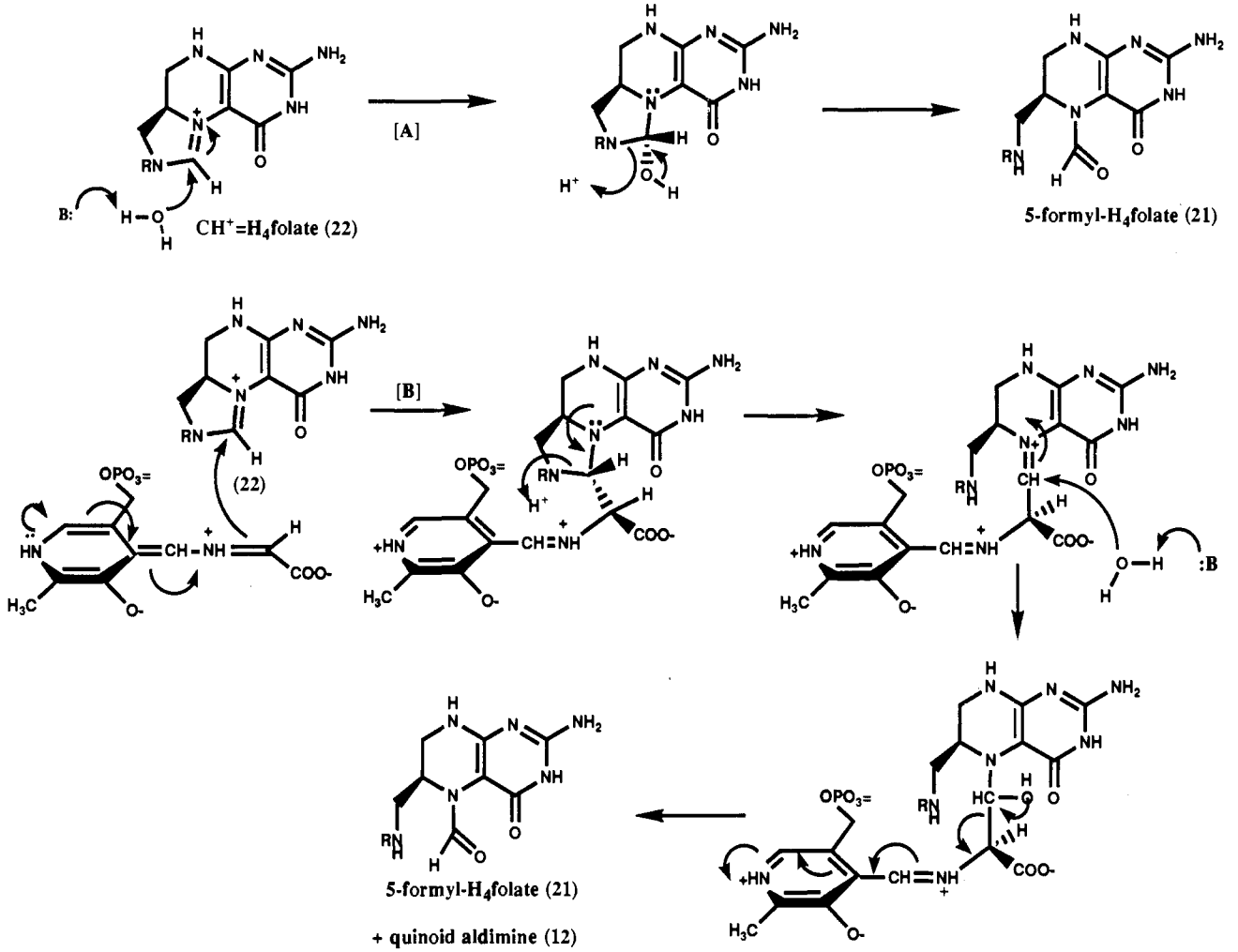
[a] Serine is depicted as forming a Schiff base imine as in Scheme I, but N^5 of H_4folate is proposed to function as a nucleophile to displace water in a reversible reaction. The covalently coupled intermediate is resolved by anti elimination of the glycine quinoid aldimine, and the 5-iminium cation so generated cyclizes to form CH_2-H_4folate. The quinoid is reprotonated to give the glycine Schiff base form of the enzyme.

by N^5 of tetrahydrofolate at the electrophilic β-carbon of the serine aldimine (13) with the loss of water. The intermediate formed (20) is directly comparable to the characterized intermediate in the transfer of a methylene group from CH_2-H_4folate to dUMP by thymidylate synthase.[25,20] Resolution of the intermediate in which the H_4folate and quinoid species are covalently coupled is accomplished by anti elimination of the quinoid anion, accompanied by the formation of the 5-iminium cation (16) of tetrahydrofolate, which presents the required *si* face to N^{10} for closure to CH_2-H_4folate. Viewed in the reverse direction, the quinoid species is able to add to the less hindered *re* face of the iminium cation, a reaction step consistent with the facial selectivities of addition to the 5-iminium cation intermediates proposed for thymidylate synthase (dUMP anion addition)[20] and methylenetetrahydrofolate reductase.[16c] The turnover is completed by the replacement of glycine by serine in the external aldimine (14).

Since free formaldehyde is not an intermediate, the reaction shown in Scheme III should proceed with complete stereospecificity, provided that aldol cleavage of the serine aldimine is not kinetically competitive, and the enzyme would not be expected to catalyze the condensation of H_4folate with formaldehyde, nor the dehydration of the carbinolamine. Lack of precedent for such a nucleophilic dehydration is one major reservation about such a scheme.

The proposed mechanisms for serine hydroxymethyltransferase lead to different predictions with respect to the facial orientation of the pyridoxal phosphate serine aldimine, which donates the one carbon unit, and the H_4folate acceptor. In Scheme I, retroaldol cleavage of the serine aldimine generates formaldehyde, which occupies a plane above and parallel to the quinoid it leaves behind. N^5 of H_4folate is presumed to trap formaldehyde by *re* face nucleophilic attack before it has an opportunity to rotate, a restriction that places N^5 in close proximity to the formaldehyde carbon in a plane above the formaldehyde. The facial orientation of the pterin is related to the nature of the elimination (syn vs anti), as depicted in Scheme I. The same orientations of H_4folate would be required for mechanisms involving nucleophilic attack of N^5 of H_4folate on the serine aldimine with elimination of the quinoid aldimine, again depending on whether the subsequent dehydration occurs by syn or anti elimination. If water is displaced by an S_N2 attack of N^5 on the β-carbon of serine in the reaction sequence of Scheme III, one would predict that N^5 would be roughly collinear with the β-carbon and the departing water. If elimination of water from the serine aldimine is the first step along the reaction path, then positioning of N^5 would be either above or below the plane of the aldimine in an

SCHEME IV. Alternative Mechanisms for the Glycine-Dependent Formation of 5-Formyl-H_4folate (21) from CH^+=H_4folate (22) Catalyzed by Serine Hydroxymethyltransferase[26a]



[a] The mechanism shown as pathway A involves hydration of CH^+=H_4folate and subsequent ring opening to yield 5-formyl-H_4folate. Although no apparent role for the glycine aldimine is indicated, it might be required to serve as a proton donor to N^{10} in the ring-opening reaction. The mechanism shown as pathway B involves nucleophilic attack of the quinoid aldimine on CH^+=H_4folate. Subsequently, ring opening and hydration of the covalently coupled intermediate occurs, followed by retroaldol cleavage to generate 5-formyl-H_4folate and the quinoid aldimine.

orientation appropriate for Michael addition to the terminal sp^2 carbon, which would fall into the plane of the aldimine to maximize overlap of the extended π-system. X-ray structural analysis of the protein with the quinoid aldimine derived from glycine and a bound N^5-substituted H_4folate derivative may therefore provide insight into the physiological sequence of events.

D. Enzyme-Catalyzed Hydrolysis of Methylidenetetrahydrofolate

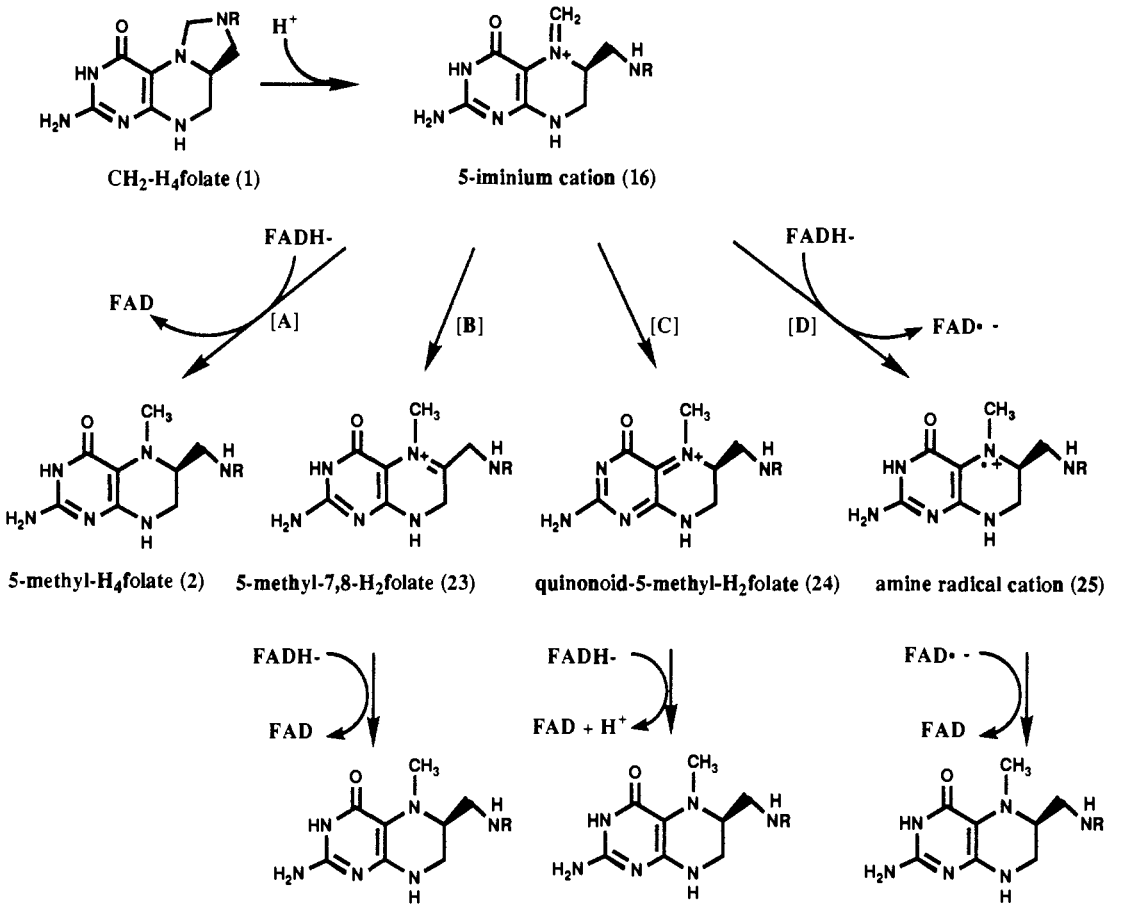
Recently, Stover and Schirch[26] have observed that serine hydroxymethyltransferase catalyzes the formation of 5-formyltetrahydrofolate (21; Scheme IV) from CH^+=H_4folate (22; Scheme IV). This reaction occurs only in the presence of glycine or D-alanine. While the requirement for glycine or D-alanine was interpreted as indicating that the aldimine serves as a general-acid catalyst in the reaction (Scheme IVA), it is also possible that glycine or D-alanine serves as a nucleophilic catalyst of the reaction (Scheme IVB). If nucleophilic participation of glycine can be demonstrated, this would support a mechanism of the type shown in Scheme III, by indicating appropriate proximity and orientation of the glycine aldimine at the active site to permit attack of the carbanion on an α-substituent at N^5 of H_4folate.

III. Methylenetetrahydrofolate Reductase

A. Comparison of the Enzymes from Prokaryotic and Eukaryotic Sources

Since the regulation and catalysis of methylenetetrahydrofolate reductase have been reviewed recently,[27] this review will stress stereochemical studies on the enzyme that were not included in those reviews and will provide only a relatively brief overview of the earlier literature. Proteins with methylenetetrahydrofolate reductase activity are found in both prokaryotes and eukaryotes but differ significantly in their properties. Enzymes from mammalian sources contain noncovalently bound FAD but lack other cofactors or metal ions.[28] The enzyme from pig liver has been purified to homogeneity[28] and shown to be a dimer of identical subunits of 77-kDa molecular mass.[29] It catalyzes the transfer of a hydride equivalent from NADPH to CH_2-H_4folate, and menadione can serve as an alternate electron acceptor.[30] In contrast, the enzymes from

SCHEME V. Possible Mechanisms for Methylenetetrahydrofolate Reductase[a]



[a] All mechanisms are presumed to commence by ring opening of CH_2-H_4folate (1) to form a 5-iminium cation (16). In pathway A, this intermediate is reduced by transfer of a hydride equivalent from the reduced enzyme-bound flavin to the exocyclic methylene group to generate CH_3-H_4folate (2). In pathway B, the iminium cation undergoes tautomerization to form 5-methyl-7,8-dihydrofolate (23), while in pathway C tautomeric rearrangement yields quinonoid 5-methyl-H_2folate (24). These species are then reduced by introduction of two electrons from reduced flavin. In pathway D, the reduction of 5-iminium cation is postulated to proceed by one-electron steps, first with donation of an electron and a proton to yield the amine radical cation (25) and then by donation of an electron to form CH_3-H_4folate.

prokaryotic sources do not react with pyridine nucleotides but are reduced by $FADH_2$ or by reduced ferredoxin.[31] The enzyme from *Clostridium formicoaceticum* has been purified to homogeneity and shown to have an $\alpha_4\beta_4$ structure.[31b] The enzyme contains 1.7 mol of FAD, 15 iron, 2 zinc, and 20 acid-labile sulfur atoms per $\alpha_4\beta_4$ octamer.[31b] At the time of writing of this review, very little mechanistic information is available on the prokaryotic enzymes, and our discussion will focus on studies with the enzyme from mammalian sources.

B. Thermodynamic and Kinetic Characteristics of the Reaction Catalyzed

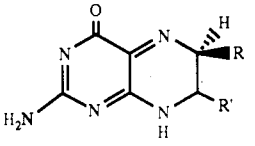
The NADPH-linked reduction of CH_2-H_4folate to CH_3-H_4folate proceeds with a free energy decrease of 9.5 kcal/mol and is essentially irreversible both in vitro and in vivo.[32] The standard reduction potential for the CH_2-H_4folate/CH_3-H_4folate couple at pH 7 is −0.130 V vs SHE,[31a] and the standard reduction potential for the enzyme-bound flavin/dihydroflavin couple at pH 7 is −0.143 V for the enzyme from pig liver.[32] Since $E^{\circ\prime}$ for the $NADP^+$/NADPH couple is −0.336 V,[33] reduction of the enzyme-bound flavin by NADPH constitutes the irreversible segment of the reaction, and the reduced flavoprotein can readily be reoxidized by high-potential electron acceptors like menadione, while the oxidized enzyme is reduced by CH_3-H_4folate.

Steady-state kinetic studies of the NADPH-linked reduction of CH_2-H_4folate to CH_3-H_4folate show parallel line kinetics, indicative of a ping-pong mechanism in which the flavoprotein is reduced by NADPH, $NADP^+$ dissociates, and then the reduced flavoprotein is oxidized by CH_2-H_4folate followed by dissociation of CH_3-H_4folate.[34] Rapid-reaction presteady-state kinetic measurements with use of a stopped-flow apparatus have established the kinetic competence of the reductive half-reaction in the absence of folate derivatives and of the oxidative half-reaction in the absence of pyridine nucleotides.[34] Thus, the reduction of CH_2-H_4folate by reduced flavin does not require participation of a bound pyridine nucleotide cofactor.

C. Possible Reaction Mechanisms

CH_2-H_4folate is the nitrogen analogue of an acetal, an aminal, and its reduction by reduced flavin poses several interesting chemical problems. It has been assumed that the first step in this reduction would be an acid-catalyzed ring opening to form a 5-iminium cation (16; Scheme V),[18,35] although direct evidence for participation of such an intermediate is lacking. Path A of Scheme V depicts direct hydride transfer from the enzyme-bound reduced flavin to the exocyclic methylene group of the 5-iminium cation. Tenable alternative

mechanisms would involve an intramolecular oxidoreduction to generate either 5-methyl-7,8-H_2folate (23; Scheme V) or 5-methyl-quinoid-H_2folate (24; Scheme V) and subsequent reduction of the H_2folate derivative. These possibilities are shown as paths B and C in Scheme V. If [6-^{3}H]-5-methyl-H_4folate is oxidized by methylenetetrahydrofolate reductase, there is no detectable release of tritium to solvent. There is also no transfer of tritium from C^6 of CH_2-H_4folate to the methyl group of CH_3-H_4folate. These observations preclude mechanisms involving a 5-methyl-7,8-H_2folate intermediate, including mechanisms in which the tritium is transferred from C^6 to the methyl group at N^5 by a 1,3 hydride shift.[35] However, it was found that the enzyme readily reduces quinoid H_2pterin derivatives (26) that lack a substituent at N^5 to the corresponding



quinoid H_2-pterin (26)

R = p-aminobenzoyl Glu or

CHOH·CH_2OH-$CH_2OPO_3^{2-}$, R' = H

or, R = R' = CH_3

H_4pterin derivatives, with values for k_{cat} and K_m that are similar to those for the physiological substrate CH_2-H_4folate.[36] These observations may reasonably be interpreted as indicating that the enzyme delivers electrons into the pterin ring of CH_2-H_4folate, rather than into the exocyclic methylene group, and they thus disfavor a mechanism involving direct hydride transfer from the flavin to the methylene group.

Mechanism C in Scheme V assumes an intramolecular redox reaction and tautomerization in which CH_2-H_4folate is converted to quinoid 5-methyl-H_2folate, which is then reduced. Alternatively, mechanism D in Scheme V assumes one-electron reduction of N^5 of CH_2-H_4folate and protonation to form an amine radical cation (25), with subsequent one-electron reduction at N^5 to generate CH_3-H_4folate. Viewed in the reverse direction, one-electron oxidation of CH_3-H_4folate would generate the amine radical cation, and such compounds show high acidity of the hydrogens attached to α-carbons compared to the parent tertiary amine.[37] Further mechanistic studies will be required to distinguish between these two possibilities, and the use of 5-deazaflavin-reconstituted enzyme, which would not readily undergo one-electron oxidation/reduction, may be helpful in determining whether reduction of CH_2-H_4folate proceeds by one- or two-electron steps.

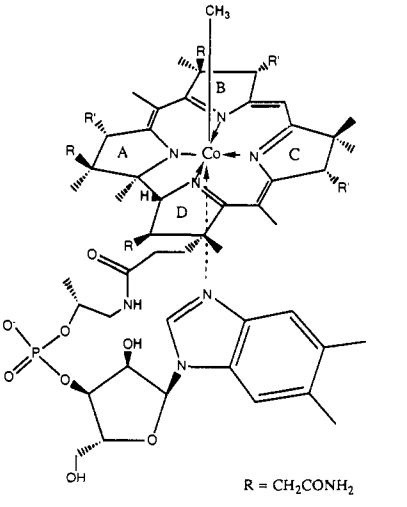
D. Stereochemical Analysis of the Catalytic Reaction

A stereochemical analysis of the enzyme-catalyzed reduction of CH_2-H_4folate to CH_3-H_4folate has now been performed.[16c] For this analysis, chiral (11*R*)-[11-^{3}H]- or (11*S*)-[11-^{3}H]CH_2-H_4folate was generated with serine hydroxymethyltransferase from pig liver and coupled to an excess of methylenetetrahydrofolate reductase in the presence of NADPH. These reactions were run in deuterated buffer, since the hydrogen incorporated into the methyl group of CH_3-H_4folate during enzymatic reduction is derived from solvent.[38] The CH_3-H_4folate product was analyzed by chemical degradation to methylamine and conversion to acetate.[16c] To ensure that the reaction conditions did not result in the racemization of CH_2-H_4folate in solution, serine hydroxymethyltransferase was coupled with an excess of methylenetetrahydrofolate dehydrogenase under identical reaction conditions, and the resultant NADPH and CH^+=H_4folate were analyzed for their tritium content. Approximately 10% racemization was observed in these control reactions. From the coupled serine hydroxymethyltransferase/methylenetetrahydrofolate reductase reaction, (3*R*)-[3-^{3}H]serine was converted to (*R*)-[*methyl*-^{3}H,^{2}H$_1$]CH_3-H_4folate and the reaction proceeded with ~75% stereospecificity.[16c] The results indicate that reduction of the methylene group of CH_2-H_4folate takes place with addition of hydrogen to the more sterically accessible face of the pteridine; this is the same face from which methylenetetrahydrofolate dehydrogenase abstracts a hydride equivalent and it is also the face attacked by enzyme-bound dUMP in the thymidylate synthase reaction.[20] These results would be consistent either with a direct transfer of hydrogen from the flavin to CH_2-H_4folate as implied by mechanisms A or D or with a general acid-catalyzed addition of a proton to the exocyclic methylene group, as implied by mechanism C in Scheme V.

IV. Methionine Synthase (Methyltetrahydrofolate-Homocysteine Methyltransferase)

A. Comparison of the Cobalamin-Dependent and Cobalamin-Independent Enzymes

Prokaryotic organisms such as the enterobacteria *Escherichia coli* and *Salmonella* have two proteins that catalyze the synthesis of methionine from homocysteine; one contains cobalamin (27) as a cofactor (the *metH*



R = CH_2CONH_2

R' = $CH_2CH_2CONH_2$

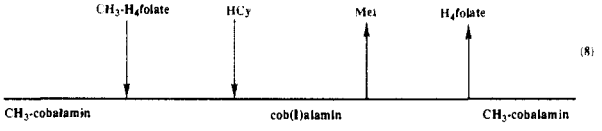
Methylcobalamin (27)

gene product), and the other is cobalamin-independent (the *metE* gene product). The activities of the two enzymes can easily be distinguished. The cobalamin-independent enzyme has a strict requirement for the polyglutamate form of the substrate CH_3-H_4folate and

requires magnesium and phosphate ion for activity.[39] The cobalamin-dependent enzyme uses monoglutamate as well as polyglutamate forms of CH_3-H_4folate and is routinely assayed with the monoglutamate. This enzyme shows an absolute requirement for AdoMet and a reducing system. Little is known about the chemical mechanism of the cobalamin-independent enzyme, but the cobalamin-dependent enzyme from *E. coli* has been cloned, overexpressed, and sequenced[40] and its mechanism has been studied extensively. Several comprehensive reviews of the work prior to 1984 have been written,[27a,41] and more recent reviews have emphasized regulation of the expression of the *metH* and *metE* genes,[42] structure–function studies on the cobalamin-dependent enzyme,[42] and the mechanistic similarities of the cobalamin-dependent methionine synthase with other cobamide-dependent methyl transferases from methanogenic and acetogenic bacteria.[43] Much less is known about the properties of the enzymes from mammalian sources, although these enzymes contain cobalamin, use the monoglutamate form of CH_3-H_4folate, and show requirements for AdoMet and a reducing system for activity in in vitro assays.[44] The available evidence suggests that the mechanisms of the cobalamin-dependent enzymes from both prokaryotic and eukaryotic sources will prove to be similar and that studies of the bacterial enzyme will be pertinent to an understanding of catalysis in both systems.

B. Catalytic Mechanism of the Cobalamin-Dependent Enzyme

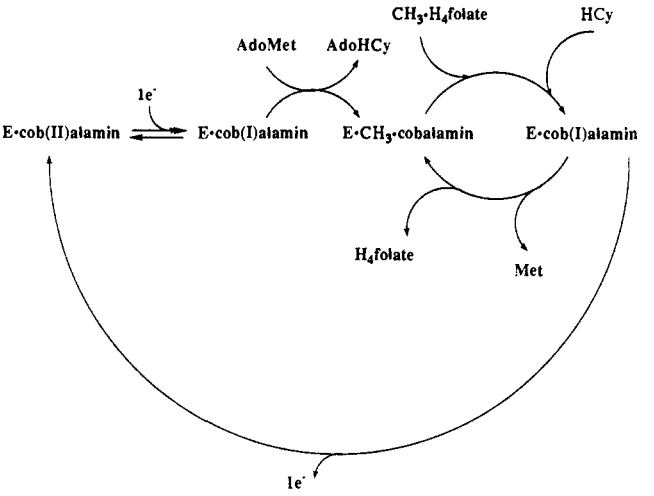
Studies from the laboratories of Weissbach, Huennekens, Taylor, and Woods (reviewed in refs 27a and 41) have established the overall mechanism for cobalamin-dependent methionine synthase shown in Scheme VI. In this mechanism, the enzyme-bound cobalamin cycles between cob(I)alamin and methylcobalamin forms, alternately being methylated by CH_3-H_4folate and demethylated by homocysteine. Rapid-reaction pre-steady-state and steady-state kinetic studies indicate that catalysis occurs by an ordered sequential kinetic mechanism as shown by eq 8.[45] The overall



(8)

transfer of a methyl group from CH_3-H_4folate to homocysteine proceeds with retention of stereochemistry at the transferred carbon,[46] as expected for a catalytic sequence involving two consecutive nucleophilic displacements. While the studies of Taylor and Hanna[47] and Fujii and Huennekens[48] provided evidence that cob(I)alamin is formed when methylated enzyme reacts with homocysteine, they did not establish the kinetic competence of cob(I)alamin. Recent investigations in our laboratory[45] established that cob(I)alamin was formed on reaction of methylated enzyme with homocysteine at 25 °C with a rate constant of 80 s^{-1}, and cob(I)alamin so generated was methylated by CH_3-H_4folate with a rate constant of ~250 s^{-1}. These rate constants are considerably faster than the turnover number of 20 s^{-1} measured under the same conditions and establish the kinetic competence of the cob(I)alamin intermediate in catalysis.

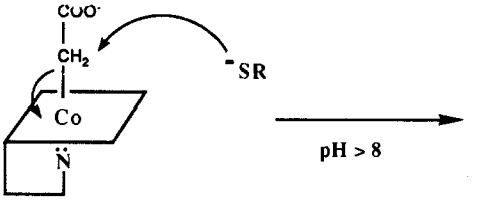
SCHEME VI. General Catalytic Scheme for the Cobalamin-Dependent Methionine Synthase Reaction[a]



[a] The enzyme-bound cobalamin cycles in catalysis between CH_3-cobalamin and cob(I)alamin. Catalysis occurs in a ternary complex, after both CH_3-H_4folate and HCy bind to methylated enzyme. Occasional intereception of the reactive cob(I)alamin by electron acceptors results in oxidation to form enzyme-bound cob(II)alamin, which is inactive in catalysis. To return the inactive enzyme to the catalytic cycle requires a reductive methylation with AdoMet serving as the methyl donor.

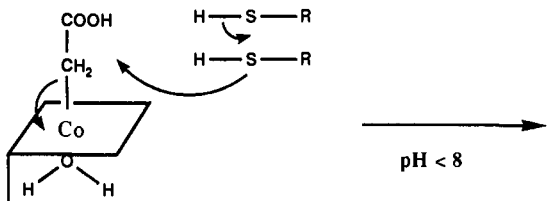
C. Model Studies for the Demethylation of Methylcobalamin by Homocysteine

There is good precedent in model systems for a nucleophilic displacement by the thiolate of homocysteine at the methyl group of methylcobalamin.[49] The rate of reaction of methylcobalamin with thiols was shown to increase as the pH was raised, with the inflection point corresponding to the pK of the attacking thiol, consistent with a nucleophilic displacement of the methyl group by the thiolate and generation of cob(I)alamin and the methyl thioether. The presence of bulky substituents on the methylene group attached to cobalt was shown to decrease the rate of nucleophilic displacement by thiolates quite markedly,[50] and this was thought to form the basis for the previously observed inhibition of methionine synthase by propyl iodide.[51] Although these reactions were conducted anaerobically, the formation of cob(I)alamin was not directly demonstrated and cob(II)alamin was observed as the product of the reaction. The formation of cob(II)alamin was attributed to the oxidation of cob(I)alamin by disulfide contaminants of the thiols employed. The reaction of (carboxymethyl)cobalamin with thiols was also studied.[50] At high pH, thiolate attack on the of (carboxymethyl)cobalamin with thiols was also studied.[50] At high pH, thiolate attack on the methylene group gave rise to *S*-(carboxymethyl) thioethers, as shown in eq 9, while at pH values where the di-



$RSCH_2COO^-$ + cob(I)alamin (9)

methylbenzimidazole substituent is protonated and no longer coordinated to cobalt ("base-off" cobalamin), thiol-dependent reductive cleavage of the (carboxymethyl)cobalamin occurs to form acetate, cob(II)alamin, and disulfide, as shown in eq 10. This reaction is



CH_3-COOH + cob(I)alamin + H^+ (10)

extremely fast (0.02 s^{-1} at pH 1.0) as compared to the rate constants observed for nucleophilic displacement by thiolates at high pH values (0.001 s^{-1} at pH 12.5). The increased reactivity of base-off cobalamin to nucleophilic attack was attributed to both steric and electronic effects.

The thermodynamic effects of nucleophilic coordination on Co–C bond strengths of methylcobalamin in neutral aqueous solution have recently been examined by Kräutler.[52] In these studies, the equilibria between methylcobalamin and cob(I)inamide or cob(II)inamide were measured, as shown in eqs 11 and 12. Cobin-

methylcobalamin + cob(I)inamide → cob(I)alamin + methylcobinamide (11)

methylcobalamin + cob(II)inamide → cob(II)alamin + methylcobinamide (12)

amides lack the dimethylbenzimidazole nucleotide that coordinates to the lower axial position of base-on cobalamin and are therefore good models for base-off cobalamins. Coordination of the nucleotide was found to stabilize the carbon–cobalt bond toward heterolytic cleavage by ~3.3 kcal/mol and to stabilize it toward homolytic cleavage by ~0.3 kcal/mol. Nucleotide coordination is also thought to have a marked effect on the fold angle along the Co–C^{10} axis of the corrin ring of cobalamin derivatives. This fold is induced due to steric hindrance between the dimethylbenzimidazole and C^5 of the corrin ring.[53] The fold angle is 15.8° in base-on methylcobalamin. In the base-off form, this steric hindrance should be relieved and a more nearly planar corrin ring conformation is expected, allowing greater access to an attacking nucleophile. Thus, studies on the structure and reactivity of alkylcobalamins strongly suggest that a base-off methylcobalamin would be the favored intermediate for heterolytic cleavage of the carbon–cobalt bond by nucleophilic attack, whether by the homocysteine thiolate or by N^5 of H_4folate.

D. Mechanistic Studies on Methylation of Cob(I)alamin by Methyltetrahydrofolate

In contrast to the half-reaction in which the methyl group of methylcobalamin is displaced by nucleophilic attack of thiolate, there is a lack of chemical precedent for the half-reaction in which cob(I)alamin is methylated with the methyl group of CH_3-H_4folate. The overall stoichiometry of this half-reaction is shown in eq 13. As indicated, the reaction involves nucleophilic

cob(I)alamin + CH_3-H_4folate → methylcobalamin + H_4folate (13)

attack by cob(I)alamin on the α-carbon of a tertiary amine. Cob(I)alamin does react with alkyl halides by a classical S_N2 mechanism,[54] but the rates of such reactions show a strong dependence on the pK_a of the leaving group, and from the limited data available we estimate a value for β_{lg} of ~−1.5. These facts suggest a transition state characterized by a high degree of bond breakage between the alkyl group and the leaving group.

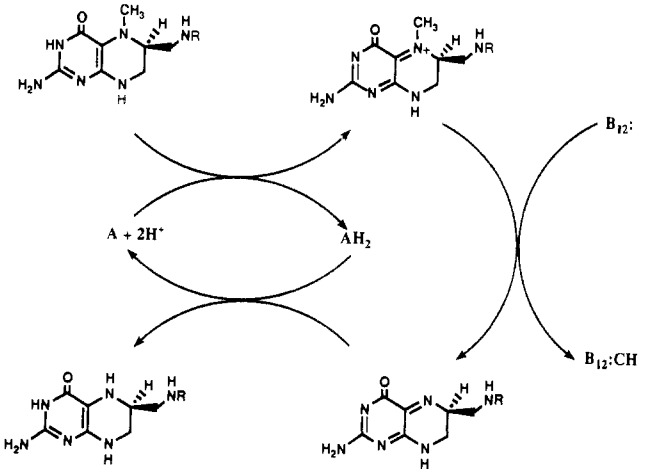
Three modes of activation of the tetrahydropteridine leaving group can be envisaged: activation by protonation at N^5, two-electron oxidation to form a dihydropteridine,[55] or one-electron oxidative activation at N^5 to form an amine radical cation. The pK_a for protonated N^5 of H_4folate is 4.82,[56] and H_4folate would be a reasonable leaving group if cob(I)alamin functioned as a nucleophile to displace the methyl group from the protonated form of CH_3-H_4folate. Two-electron oxidation to yield 5-CH_3-7,8-H_2folate would generate an H_2folate leaving group with a pK_a of 3.84.[57] However, such an oxidative mechanism is rendered unlikely by the failure to see release of tritium when CH_3-[6,7-^{3}H]H_4folate was used as the substrate for methionine synthase.[55a] Alternative mechanisms for oxidative activation of CH_3-H_4folate prior to methyl transfer might involve formation of the quinonoid 5-CH_3-H_2folate (**24**; Scheme V) or one-electron oxidation to form an amine radical cation at N^5 (**25**; Scheme V). Exact values for the acid dissociation constants of N^5 in these two compounds are not available. Oxidative activation of CH_3-H_4folate would require the presence of a high-potential electron-accepting group on methionine synthase, and studies thus far have failed to identify such a group. $E^{\circ\prime}$ for 6,6,7,7-tetramethyl-H_2pterin/6,6,7,7-tetramethyl-H_4pterin is +0.105 V vs SHE.[59] A mechanism for two-electron oxidative activation of CH_3-H_4folate in the methionine synthase reaction is shown in Scheme VII.

E. Role of Adenosylmethionine in Enzyme Activation

During in vitro catalysis of the methionine synthase reaction, the catalytic intermediate cob(I)alamin is slowly oxidized to an inactive enzyme form, cob(II)alamin. In order to return cob(II)alamin to the catalytic cycle, AdoMet and a reducing system must be present in addition to the substrates HCy and CH_3-H_4folate. The activation of cob(II)alamin is depicted as a reductive methylation in Scheme VI, with AdoMet required as the methylating agent for conversion of cob(II)alamin to methylcobalamin, although the substrate, CH_3-H_4folate, is capable of rapid reaction with cob(I)alamin in the catalytic cycle. Thermodynamic analysis of the reductive methylation, described below, has established that the exergonic methyl transfer from AdoMet to cob(I)alamin is used to drive a very endergonic reduction of cob(II)alamin to cob(I)alamin in an example of a coupled electron-transfer reaction.

In vitro activation of the enzyme is achieved with AdoMet and dithiothreitol or dithioerythritol and aquocobalamin or with AdoMet, FAD or FMN, hy-

SCHEME VII. Mechanism for Oxidative Activation of the Methyl Group of CH_3-H_4folate To Permit Nucleophilic Displacement by Cob(I)alamin[a]



[a] In this mechanism, two-electron oxidation of CH_3-H_4folate results in formation of quinoid 5-methyl-H_2folate and the concomitant reduction of an unidentified enzyme-bound group (e.g., a redox-active disulfide). Following nucleophilic displacement of the methyl group by cob(I)alamin, the quinoid H_2folate is reduced to H_4folate.

drogen gas, and platinum oxide.[60] Flavoproteins, present in crude extracts from *E. coli*, were also shown to catalyze pyridine–nucleotide-dependent reduction of methionine synthase.[60a,61] A pair of flavoproteins have been purified from *E. coli* K-12 that mediate NADPH-dependent reduction of methionine synthase.[61] These flavoproteins have been designated the R and F proteins. The R protein has properties similar to those of ferredoxin-$NADP^+$ oxidoreductases, and the enzyme-bound FAD undergoes two-electron reduction in the presence of NADPH. The F protein contains noncovalently bound FMN and exhibits properties similar to those of flavodoxins. These proteins are small, highly acidic electron-transfer proteins that can substitute for ferredoxin in iron-deficient growth media in nitrogen-fixing bacteria and blue green algae and mediate the light-dependent reduction of $NADP^+$ by transfer of electrons to the flavoenzyme ferredoxin-$NADP^+$ oxidoreductase.[62] Flavodoxin has been isolated and purified from *E. coli* and has been shown to play a role in the AdoMet-dependent reductive activation of pyruvate-formate lyase.[63] In the activation of methionine synthase, flavodoxin is reduced by electron transfer from the R protein[61b] and transfers electrons to methionine synthase in its inactive cob(II)alamin form.[64] Cob(I)alamin is never observed as an intermediate in the reduction, which is rigorously coupled to methylation of cob(I)alamin by AdoMet.[48,64]

The standard reduction potential for the cob(II)alamin/cob(I)alamin couple in methionine synthase has been measured by a spectroelectrochemical titration in which the reduction was monitored by observing the disappearance of the EPR signal associated with cob(II)alamin.[64] This potential was found to be −0.526 V vs SHE. From the EPR signal of the enzyme-bound cob(II)alamin, it was evident that the cobalamin was in the base-on conformation, in which the appended dimethylbenzimidazole nucleotide is coordinated to cobalt in the lower axial position. The potential of the enzyme-bound cofactor is more positive than that of the base-on cofactor in aqueous solution, which is −0.61 V at pH 7.[65] However, the potential is still too negative for appreciable formation of cob(I)alamin when the enzyme is at equilibrium with the reductants employed for in vitro activation: dithiotreitol ($E^{\circ\prime}$ = −0.32 V),[66] NADPH ($E^{\circ\prime}$ = −0.336 V),[33] or flavodoxin hydroquinone ($E^{\circ\prime}$(FMNH•/$FMNH_2$) ≈ −0.455 V).[63a]

The apparent midpoint potential for reduction of enzyme-bound cob(II)alamin was also measured in the presence of CH_3-H_4folate. The equilibrium established under these conditions is shown in eqs 14 and 15. The

$$\text{cob(II)alamin} \rightleftarrows \text{cob(I)alamin} \quad (14)$$

$$\text{cob(I)alamin} + CH_3\text{-}H_4\text{folate} \rightleftarrows \text{methylcobalamin} + H_4\text{folate} \quad (15)$$

shift in the apparent standard potential measured under these conditions reflects the standard free energy for reaction 15. Titrations in the presence of CH_3-H_4folate were shown to be reversible, and the apparent midpoint potential was −0.45 V. From these results a $\Delta G^{\circ\prime}$ for the methyl transfer of eq 15 of −0.09 kcal/mol was calculated.

If cob(II)alamin was titrated in the presence of AdoMet, rather than CH_3-H_4folate, the reduction was no longer reversible, but the kinetics of disappearance of cob(II)alamin were a function of the applied potential. Even at an applied potential of −0.082 V vs SHE, conversion of cob(II)alamin to methylcobalamin proceeded to completion in the presence of AdoMet. From these data, a standard free energy decrease of more than 9 kcal/mol was calculated for the methyl transfer shown in eq 16.[64] The requirement for AdoMet for reductive

$$\text{cob(I)alamin} + \text{AdoMet} \rightarrow \text{methylcobalamin} + \text{AdoHCy} \quad (16)$$

activation in vitro is thus a thermodynamic necessity. The free energy decrease of the methyl transfer of reaction 16 is sufficient to drive the highly endergonic reduction of cob(II)alamin to cob(I)alamin at the potentials provided by the reducing systems employed for in vitro activation. Once methylcobalamin is formed, the catalytic cycle regenerates cob(I)alamin by dealkylation rather than by reduction, and reductive reactivation is only required when the enzyme is disabled by adventitious oxidation of cob(I)alamin.[61b]

F. Questions about the Mechanism of the Cobalamin-Independent Enzyme

If the nucleophilic displacement of a methyl group from a protonated tertiary amine is a formidable task, the cobalamin-dependent methionine synthase is at least equipped with an extremely powerful and very weakly basic nucleophile in cob(I)alamin. The mechanism by which CH_3-H_4folate is activated for methyl transfer by the cobalamin-independent methionine synthase is of great interest. Admittedly, the turnover number for the cobalamin-independent enzyme, 14 min^{-1} at 37 °C,[39] is very much lower than that for the cobalamin-dependent enzyme, which is ∼1400 min^{-1}.[67] Does this enzyme catalyze direct nucleophilic attack by homocysteine thiolate on CH_3-H_4folate? We plan to determine the stereochemistry associated with the methyl transfer catalyzed by this enzyme, in order to understand better the biological strategies available for

activation of saturated substituents of tertiary amines.

V. Summary

We have attempted to summarize the recent developments in our understanding of the mechanisms of three enzymes that sequentially catalyze the incorporation of the β-carbon of serine into methionine. One formal problem common to each of these three enzymes is the activation of an sp^3 substituent attached to N^5 of H_4folate either for transfer to the next acceptor or for abstraction of a hydride equivalent. In the case of serine hydroxymethyltransferase, the problem is most clearly evident when the reaction is viewed in the direction of the conversion of CH_2-H_4folate and glycine to H_4folate to serine (Scheme I). Here, the sp^3 methylene group of CH_2-H_4folate (1) is first assumed to be converted to a 5-iminium cation (16) by antiperiplanar ring opening. Hydration would yield the N^5-carbinolamine (17 or 18), which equilibrates with free formaldehyde. Formaldehyde possesses an activated sp^2 center that could readily hydroxymethylate the glycine quinoid species. Hence, one mechanistic pathway for the activation of CH_2-H_4folate is by generation of an sp^2 center at the transferred carbon. However, recent experiments showing that this transfer occurs with a high degree of stereospecificity place severe spatial and mechanistic restrictions on the allowed pathways for transfer, and we have suggested other potential pathways consistent with this result. An attractive alternative shown in Scheme III involves utilization of the 5-iminium cation directly as the activated carbon intermediate for nucleophilic attack. This reaction step is precedented in the thymidylate synthase mechanism and also postulated for methylenetetrahydrofolate reductase.

In the latter case, activation is as potentially straightforward as formation of the 5-iminium cation (16), which could accept a hydride equivalent from a reduced flavin donor (path A, Scheme V). Indeed, stereochemical analysis of chiral CH_3-H_4folate product shows 75% stereospecificity in delivery of a hydron to the *re* face of the proposed imine, a result consistent with hydride transfer to the 5-iminium cation. Alternatively, it can be noted that the exocyclic 5-iminium cation is a tautomeric form of the endocyclic quinonoid species, and delivery of electrons to the pteridine ring system is also feasible (paths B–D, Scheme V). The ability of reduced flavin to act as a one-electron donor suggests that amine radical cations (25) should be considered as activated intermediates.

Finally, methyl transfer from CH_3-H_4folate to homocysteine via the cob(I)alamin of methionine synthase requires conversion of the folate residue to a good leaving group. We have considered activation strategies in which N^5 becomes positively charged so that a nucleophilic transfer of the methyl substituent leaves behind a neutral species. General-acid protonation of N^5 would give H_4folate as the departing species. Alternatively, transient oxidation of the folate residue, either by one- or two-electron processes, would result in an activated methyl group attached to a positively charged nitrogen. Following methyl transfer, return of electrons to the flavin would be accompanied by reprotonation of N^5. However, no evidence for this putative electron depot has been accumulated, nor is there evidence for radical intermediates during turnover.

Taken together, these formal mechanisms constitute pathways by which one-carbon units are proposed to be activated for transfers to and from H_4folate and for oxidoreductions of exocyclic substituents of H_4folate, and this review has evaluated possible catalytic roles for folates in facilitating these transformations.

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Registry No. Serine hydroxymethyltransferase, 9029-83-8; methylenetetrahydrofolate reductase, 9028-69-7; methyltetrahydrofolate-homocysteine methyltransferase, 9033-23-2.

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(1) Abbreviations used: H_4folate, 5,6,7,8-tetrahydrofolate; CH_2-H_4folate, 5,10-methylenetetrahydrofolate; CH_3-H_4folate, 5-methyltetrahydrofolate; AdoMet, *S*-adenosylmethionine; AdoHCy, *S*-adenosylhomocysteine; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate; CH^+=H_4folate, 5,10-methylidenetetrahydrofolate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; $NADP^+$, oxidized nicotinamide adenine dinucleotide phosphate; FAD, flavin adenine dinucleotide.
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