azido-cytochrome c as with all other derivatives. Using the reported g values for azido-cytochrome c[44] to calculate the dipolar shift, an overall 40% increase in total spin density is estimated for OPN_3.

The N-acetyl-DL-methionine-OP complex differs in dipolar shift as well as contact shift from cytochrome c. Nevertheless there is a 29% increase in overall spin density in the model as compared to cytochrome c.

This analysis demonstrates that the actual amount of increased electron spin density in the model over the protein is highly dependent on the axial ligand. Part of this dependence may be due to changes in tertiary structure of the protein from steric interactions with bulky ligands like pyridine. The heme-protein contacts would be more important for such bulky ligands. Cyanide, a smaller ligand, probably perturbs the tertiary protein structure less, resulting in much smaller overall differences between the model and the protein. However, even though azide is a small ligand, the estimated differences are large. The asymmetry in spin density among the four pyrrole rings, illustrated by the range of heme methyl contact shifts, is more pronounced in cytochrome c than OPMET. This suggests that additional structural differences between the model and the protein exist which influence the spin-density distribution. Aside from the absence of protein structure in OPMET, the only other factor which is expected to be different in the model system is methionine orientation. The tertiary structure of the protein imposes a rigid methionine orientation. This restraint is absent in the model. Comparison of the CD spectra of OPMET and cytochrome c supports this concept since large differences in methionine orientation are apparent. The more asymmetric distribution of spin density in cytochrome c over OPMET is therefore associated with the specific orientation of methionine in the protein.

Although heme methyl group assignments are not available for OPMET, the pattern of shifts is probably the 5, 8, 1, 3, pattern found for all the other OP complexes, if methionine rotates freely. In cytochrome c, however, the sulfur lone pair orbital is pointed toward pyrrole ring IV as noted previously by Wüthrich. The effect of the lone pair interaction with the $d\pi$ orbitals is to direct the maximum spin density to the 8- and 3-methyl groups. In the model system, the sulfur lone pair can interact equally with both $d\pi$ orbitals as it rotates. Spin delocalization is therefore more isotropic, which results in a smaller spread of contact shifts in OPMET.

It is attractive to speculate that the protein, through heme-amino acid contacts, actually directs electron spin density to specific pyrrole rings. However, the dramatic contrast between the 8, 3, 5, 1 pattern of native cytochrome c and the 5, 8, 1, 3 pattern for the complexes reported here indicates that the orientation of methionine probably exerts the *primary* influence on electron spin distribution in native cytochrome c.

Stereochemical Analysis of γ-Replacement and γ-Elimination Processes Catalyzed by a Pyridoxal Phosphate Dependent Enzyme

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Contribution from the Departments of Chemistry and Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received December 23, 1980*

Abstract: (Z)- and (E)-[4-^{2}H]Vinylglycine samples have been synthesized and used to determine the stereochemical outcome of a pyridoxal phosphate dependent bacterial enzyme cystathionine γ-synthase. For the first time both the γ-replacement mode, vinylglycine or O-succinylhomoserine to cystathionine, and the γ-elimination mode, vinylglycine or O-succinylhomoserine to α-ketobutyrate, have been solved stereochemically. The diastereomeric [4-^{2}H]cystathionines produced enzymically in the γ-replacement mode were degraded to [4-^{2}H]homoserines, the absolute chiralities were determined, and then the compounds were succinylated. The O-succinyl[4-^{2}H]homoserines were then processed enzymically to [4-^{2}H]cystathionines enzymically, and the stereochemical course of the first half-reaction was thereby determined.

Pyridoxal-P (PLP), the coenzyme form of vitamin B_6, is a required cofactor for enzyme-mediated transformations at the α, β, and γ carbons of α-amino acids and acts to facilitate fluxes by forming stabilized substrate-carbanion equivalents.[1a,b] Amino acids substituted at both the β and γ carbons with potential leaving groups undergo either net elimination, an internal redox reaction to release leaving group and ultimately produce α-keto acid product, or net replacement to yield an amino acid product with a new β or γ substituent, respectively. Reactions at the β carbon are obviously enabled by α-carbanion equivalents. γ-Carbon eliminations or replacements, on the other hand, represent reactions at a formally unactivated carbon center and so pose more of a chemical challenge to the enzymic catalysts.

In fact, the small subset of the pyridoxal-P-linked enzymes that act at the γ carbon of amino acid substrates first make stabilized α-carbanion equivalents then β-carbanion equivalents for anchimeric assistance to γ-substituent departure and so abstract two substrate hydrogens (C_α, C_β).[2] This process yields a fully conjugated intermediate (eq 1a of Scheme I) that has the ambident reactivity at the γ carbon to serve as common precursor to both elimination and replacement products (eq 1b) in the second half-reactions. The elimination sequence i involves reaction of C_4 (γ) of substrate equivalent as nucleophile toward a proton, hydrolytic release of the enamine, and ketonization; the replacement sequence ii features attack of an incoming cosubstrate nucleophile, y', on C_4 as the electrophilic center, followed by stereo- and regiospecific protonation at C_3 (β) then C_2 (α) carbons to unravel the intermediate to the product amino acid.

While the stereochemical outcome of various enzymatic β replacements and β eliminations have been detailed in the last few years,[3] no analysis of the more complex and difficult γ-elimination

(1) (a) Snell, E., Dimari, S. In "The Enzymes", 3rd ed.; Boyer, P., Ed.; Academic Press: New York, 1970; Vol. 2, p 335. (b) Walsh, C. In "Enzyme Reaction Mechanisms"; W. H. Freeman: San Francisco, 1979; Chapter 24.

(2) Davies, L.; Metzler, D. In "The Enzymes", 3rd ed.; Boyer, P., Ed.; Academic Press: New York, 1972; Vol. 17, p 42.

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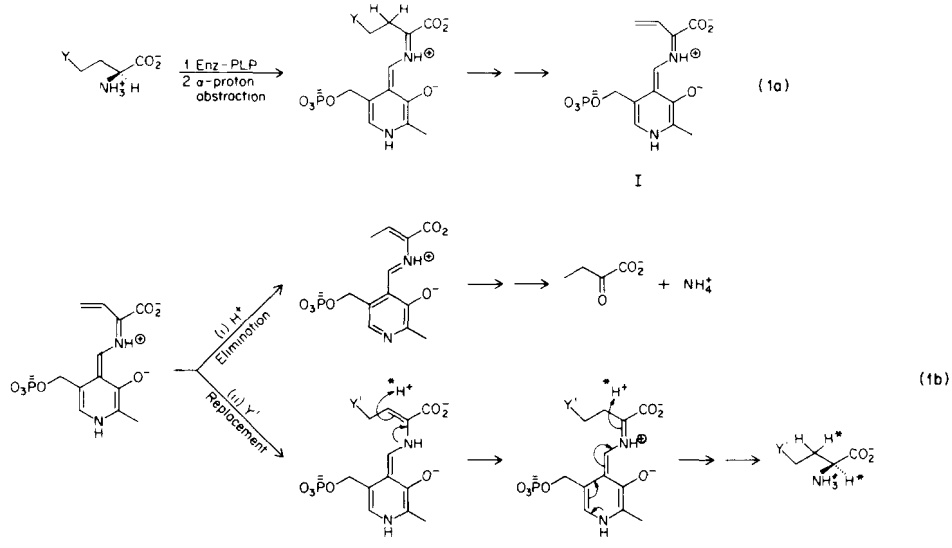
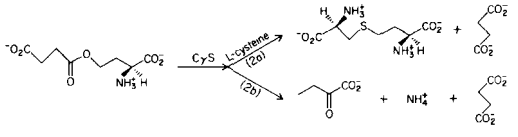
Scheme I



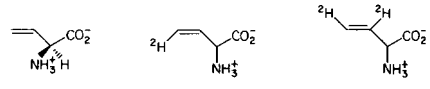
Scheme II



or γ-replacement sequences have been reported. In this paper we provide the first report of both elimination and replacement outcomes, catalyzed by a key enzyme in bacterial biosynthesis of the essential amino acid L-methionine. Cystathionine γ-synthase converts a functionalized γ-oxo substrate, *O*-succinyl-L-homoserine, to a γ-sulfur amino acid product, LL-cystathionine, the *O*-succinyl moiety replaced with the sulfur nucleophile of cosubstrate L-cysteine (eq 2a) of Scheme II). Cystathionine has the γ-sulfur atom characteristic of the eventual end product L-methionine and is subsequently processed biosynthetically to homocysteine and then methylated on sulfur. In the absence of cosubstrate L-cysteine, the γ-synthase catalyzes γ elimination at 20% the normal V_{max} and generates α-ketobutyrate, ammonia, and succinate (eq 2b) so both outcomes are amenable to study with this one enzyme.[5a,b]

If one divides the overall reaction process into two halves according to eq 1a and 1b, the stereochemical problems of the first half involve determination of (a) which prochiral β-H is abstracted, (b) what is the nature of the β-H, γ-y elimination, syn or anti, and so (c) what is the geometry, cis or trans (e.g., with 4-monodeuterio), of the key conjugated intermediate I? In the second half-reactions of eq 1b, the elimination mode i requires determination of (d) to what face (*si* or *re*) at C_4 is the proton added and so (e) what is the chirality of the C_4 methyl group in α-ketobutyrate produced; (f) the chirality of protonation at C_β is also determinable. In the replacement mode ii, there is the analogous question (g) of the face of addition of incoming nucleophile y' and (h) the chirality of reprotonation at C_β. When we began our experiments it was known (from a preliminary communication) only that the enzyme was reported to abstract the *pro-R* hydrogen at C_β (point a).[6]

Our strategy for resolution of the remaining stereochemical problems began with testing of the expectation that I was indeed the key intermediate by noting that it was merely the *p*-quinoidal form of the α anion of a PLP–vinylglycine adduct. We established



II III IV

that L-vinylglycine, II, was indeed chemically and kinetically competent to serve as alternate substrate for the pure cystathionine γ-synthase of *Salmonella*, partitioning identically to the same products generated by physiological substrate *O*-succinylhomoserine.[7] Simple α-H abstraction from vinylglycine–PLP indeed allows the unsaturated four-carbon amino acid to mesh into the normal reaction flux.

We have now synthesized (*Z*)-DL-[4-^{2}H]vinylglycine (III) and (*E*)-DL-[3,4-$^2H_2$]vinylglycine (IV) samples and have used them to enter the reaction sequence at the halfway point and to solve the stereochemistry of the second half-reactions (eq 1b) first, in *both* the elimination and replacement modes. For the elimination mode i (eq 1b), parallel enzymic incubations with *E* or *Z* substrate isomers were conducted in ^3H_2O to yield α-ketobutyrates, chiral in the C_4 methyl groups by virtue of ^{1}H,^{2}H,^{3}H substitution. Stereochemical determination was carried out by the acetate kinase, malate synthase, fumarase chiral methyl group methodology[8a,b] after degradation of α-ketobutyrate products to acetates.

Solving the set of stereochemical questions of the second half-reaction replacement mode ii (eq 1b) was considerably more complex. The two [4-^{2}H]cystathionines generated from (*E*)- and (*Z*)-deuteriovinylglycines in the presence of L-cysteine have been degraded by combined enzymic and chemical procedures to L-[4-^{2}H]homoserines. Since the absolute chirality of such homoserines had not been determined unambiguously before, we resolved that question by generating authentic (4*R*)- and (4*S*)-L-[4-^{2}H]homoserine samples with *E. coli* homoserine dehydrogenase and assigning chirality again by combined enzymic and chemical degradation to 3-hydroxypropionyl benzyl esters which proved to be substrates for alcohol dehydrogenase, an enzyme known to be *pro-R* specific at the alcoholic carbon of its substrates.[9]

The degradation of cystathionines to L-[4-^{2}H]homoserines of determined absolute chirality had now, of course, set up solution of the stereochemical problems in the first half-reaction of cys-

(3) Vederas, J.; Floss, H. *Acc. Chem. Res.* **1980**, *13*, 455.

(4) Flavin and colleagues, studying enzymic exchange at the prochiral β hydrogens of L-homoserine, noted that *both* hydrogens were exchangeable albeit at disparate rates, with H_R (upfield signal) exchange some 100-fold faster than H_S. Total washout of deuterium frqm C_3 of vinylglycine suggests that the product cystathionine may be in exchange equilibrium *before* release from the active site.

(5) (a) Guggenheim, S.; Flavin, M. *J. Biol. Chem.* **1969**, *244*, 3722; (b) Guggenheim, S.; Flavin, M. *Ibid.* **1971**, *246*, 3562.

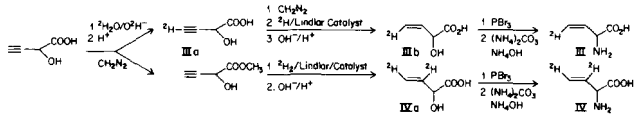
(6) Coggiola, D.; Fuganti, C. *Experientia* **1977**, *33*, 847.

(7) Johnston, M.; Marcotte, P.; Donovan, J.; Walsh, C. *Biochemistry* **1979**, *18*, 1729.

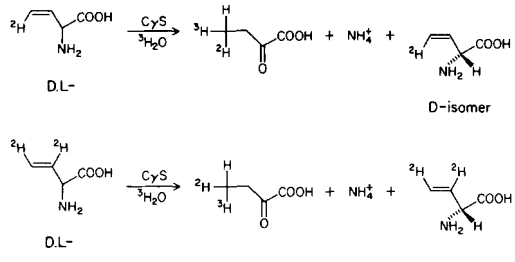
(8) (a) Cornforth, J.; Redmond, J.; Eggerer, H.; Buckel, W.; Gutschow, G. *Nature* (London) **1969**, *221*, 1212; (b) Luthi, J.; Retey, J.; Arigoni, D. *Ibid.* **1969**, *221*, 1213.

(9) Jones, J. B. In "Techniques of Chemistry"; Weissberger, A., Ed.; Wiley: New York, 1976; Vol. 10, p 107.

Scheme III



Scheme IV



tathionine γ-synthase action (eq 1a). Chemical succinylation to *O*-succinyl-L-[4-^{2}H]homoserines and enzymic conversion to chiral, diastereomeric [4-^{2}H]cystathionines allows determination of overall reaction geometry by 270-MHz NMR analysis. This in turn fixes the stereochemical outcomes of the first half-reaction and the relative and absolute geometries of the intermediates.

We report these results in this paper. Preliminary communications of a portion of these investigations have appeared recently in this journal.[10a,b]

Results

Preparation of Vinylglycines Specifically Deuterated at Carbon 4. To implement the strategy of using vinylglycine as a stereochemical probe that could be sent into mid-catalytic stream of cystathionine γ-synthase catalytic cycles, we required samples of the *E*-4-^{2}H and *Z*-4-^{2}H geometric isomers. These syntheses were effected as outlined in Scheme III. The *Z* isomer was produced (on half-gram scale) by introduction of deuterium from solvent deuterated water via exchange of the acetylide ion of 2-hydroxy-3-butynoate at p^{2}H of 9.5. Two cycles of exchange lead to >98.5% deuteration. Purification as the methyl ester was followed by controlled partial reduction with hydrogen gas and Lindlar catalyst to yield only the *Z* isomer of [4-^{2}H]vinylglycolate, which was converted to *Z*-DL-[4-^{2}H]vinylglycine (III) in three steps. A similar sequence, with omission of the deuterated water exchange and use of deuterium gas in the acetylene to olefin reduction step, produced (*E*)-DL-[3,4-$^2H_2$]vinylglycine (IV). NMR analysis indicated no contamination of either geometric isomer by the other. The DL mixture at carbon 2 was of no concern since the enzyme is specific for the 2*S* (L) isomer only. Also, the presence of deuterium at C_3 of the (*E*)-vinylglycine sample presented no problems since the enzyme washed out this deuterium into solvent during the course of catalysis (vide infra).[4]

γ-Elimination Stereochemical Outcome from Vinylglycines. In the absence of cosubstrate L-cysteine, *O*-succinyl-L-homoserine gives α-ketobutyrate, NH_4^+, and succinate, via the γ-elimination reaction mode. L-Vinylglycine similarly yields the identical products in what is, for this substrate, a net 1,3-allylic isomerization sequence from vinylglycine–PLP adduct to initial aminocrotonate–PLP product. In preliminary studies with DL-[2-^{3}H]vinylglycine we detected *no* significant 1,3-*intra*molecular proton transfer, i.e., no 2-hydroxy[4-^{3}H]butyrate on in situ enzymic reductive trapping of 2-ketobutyrate with lactate dehydrogenase and NADH.

To determine chirality at the C_4 methyl group of the 2-ketobutyrate product, enzymic incubations with (*E*)- and (*Z*)-[4-^{2}H]vinylglycines were conducted in ^3H_2O to generate some product molecules with all three isotopes of hydrogen at C_4 (Scheme IV). These molecules containing the ^{1}H,^{2}H,^{3}H methyl group were subjected to Kühn–Roth degradation immediately to prevent nonenzymic enolization (slow on the incubation time scale) from scrambling chirality at C_4. The resulting chiral acetates from the two parallel incubations were then rigorously purified from any residual propionate by conversion to the bromophenacyl ester, thin-layer chromatography, deesterification, and, finally, crystallization of sodium acetate. The acetate samples were mixed with [U-^{14}C]acetate to allow more sensitive counting of ^{3}H/^{14}C ratios in subsequent assays. The two acetate samples were then analyzed for chirality in the laboratory of Professor Heinz Floss at Purdue University, using the standard four-enzyme couple of acetate kinase, phosphotransacetylase, malate synthase, and fumarase.[8] Completely pure (*R*)-acetate samples release 21% of their tritium in this assay, the (*S*)-acetates show 79% release at fumarase equilibrium, and achiral samples, of course, show a 50:50 distribution.[8,11] Table I indicates that acetate samples of appropriately opposite chirality were obtained from the two 4-deuteriovinylglycine isomers. The *Z*-4 isomer yields (2*S*)-acetate and so (4*S*)-ketobutyrate, while the (*E*)-[4-^{2}H]vinylglycine molecules are processed to (4*R*)-ketobutyrate. Cystathionine γ-synthase is chiral in its action; the less than 100% chiral purity values could represent some loss of stereochemical control by the enzyme but more likely represents some loss of chiral integrity in the samples during workup.

Table I. Chiral Methyl Analysis of the γ-Elimination Mode Products

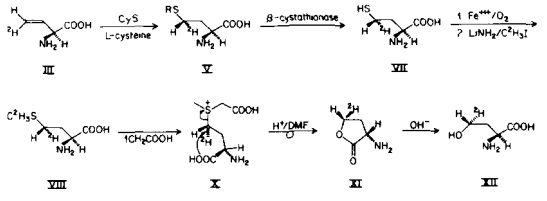
	^{3}H/^{14}C ratio in acetate	^{3}H/^{14}C ratio in malate[a]	% tritium retention	chirality assignment[11]
(*Z*)-[4-^{2}H]-vinylglycine (III) (III)	3.87	1.29	34	*S*
(*E*)-[3,4-$^2H_2$]-vinylglycine (IV)	1.95	1.21	62	*R*

[a] After fumarase action.

Table II. 270-MHz NMR Chemical Shifts (Hz) of Proton at C_4 Position of Degradation Products

	(*E*)-[4-^{2}H]-vinylglycine	(*Z*)-[4-^{2}H]-vinylglycine
cystathionine	687.9	696.5
methionine	858.0	855.5
homoserine	1027.5	1029.3

Scheme V



Results of Enzymic Incubations. Enzymic incubations for γ-replacement-type reaction with vinylglycines contained 100 mM L-cysteine in overnight incubations and yielded, from 1 mmol of (*Z*)-[4-^{2}H]vinylglycine, 102 mg of a LL-[4-^{2}H]cystathionine; (*E*)-[4-^{2}H]vinylglycine similarly generated 67 mg of diastereomeric LL-[4-^{2}H]cystathionine, purified as described in the Experimental Section. Table II shows the diastereomeric location of deuterium atoms at C_4 in each cystathionine sample V (from (*Z*)-[4-^{2}H]vinylglycine) and VI (from (*E*)-[4-^{2}H]vinylglycine).

Absolute chirality assignment required degradation to homoserines via the route indicated in Scheme V. The first step was enzymatic and involved homogeneous β-cystathioninase from *E. coli*,[13] a PLP-dependent enzyme specific for catalysis on the

(10) (a) Chang, M. N.; Walsh, C. *J. Am. Chem. Soc.* **1980**, *102*, 2499; (b) Chang, M. N.; Walsh, C. *Ibid.* **1980**, *102*, 7368.

(11) Cornforth, J. W.; Redmond, J. W.; Eggerer, H.; Buckel, W.; Gutschow, C. *Eur. J. Biochem.* **1970**, *14*, 1.

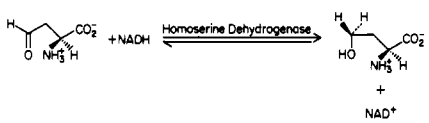
Table III. 270-Hz NMR Chemical Shift (Hz) of Proton at C_4 Position of L-Homoserines Generated by L-Homoserine Dehydrogenase Action

(4*R*)-L-[4-^{2}H]-homoserine from [4-^{2}H]aspartate semialdehyde	(4*S*)-L-[4-^{2}H]-homoserine from (4*S*)-[4-^{2}H]NADH
1027.5	1029.3

three-carbon arm only of cystathionine, and provided mild, quantitative conversion of the seven-carbon thiol ether diamino acid to the four-carbon homocysteine VII without effect at the important C_4 methylene center of cystathionine. Standard chemical steps led methionine VIII to homoserine lactone XI via the one inversion at C_4 in the sequence and then to L-[4-^{2}H]-homoserine samples XII and XIII. The chiral integrity of the two parallel samples (from (*E*)- and (*Z*)-vinylglycines) could be monitored by 270-MHz NMR analysis at each step, and the γ-proton chemical shifts are collected in Table II, confirming no effect of the degradation sequence on chirality at the key carbon center.

Absolute Chirality of [4-^{2}H]Homoserine Diastereomers. The degradation of cystathionine samples to homoserine samples had two purposes. First, we had in mind a method for absolute chirality assignment at the C_4 alcoholic carbon of homoserine, and second, the diastereomeric L-[4-^{2}H]homoserines could serve as substrates, once succinylated, for the overall cystathionine γ-synthase γ-replacement sequence (sequence of eq 1b).

While the above experiments were in progress we focused on the preparation of reference standards of (4*R*)- and (4*S*)-L-[4-^{2}H]homoserines and turned to an enzymic synthesis that was carried out by a bifunctional enzyme from *E. coli*, aspartokinase-L-homoserine dehydrogenase. The second activity is the useful one here, catalyzing the reversible interconversion of aspartate semialdehyde and L-homoserine at the expense of NADH oxidation[14] (eq 3).

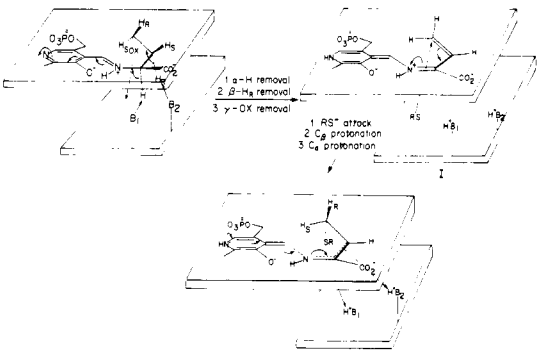


(3)

We established that this dehydrogenase uses the 4-*pro-S* hydrogen of NADH in the redox step and so could use (4*S*)-[4-^{2}H]NADH to prepare 10-mg quantities of a [4-^{2}H]homoserine for 270-MHz NMR analysis. With the assumption of chiral action by the enzyme, the other [4-^{2}H]homoserine diastereomer ought to be available from [4-^{2}H]aspartate semialdehyde and NADH. This goal was realized, and the deuterated aldehyde was prepared by ozonolysis of 4,5-dideuterioallylglycine, in turn generated by partial reduction of propargylglycine with deuterium gas and Lindlar catalyst in deuterated water. The 270-MHz NMR data (Table III) of the two L-[4-^{2}H]homoserine enzymic products proved they were indeed diastereomeric and homoserine dehydrogenase is indeed catalyzing chiral reduction of the aldehyde functionality. To use these samples as reference standards it remained to assign absolute chirality to the C_4 alcoholic group in such monodeuteriohomoserines. This, in turn, meant solving the stereochemical outcome of homoserine dehydrogenase action, since that fact was unreported.

Our strategy was to use (4*S*)-[4-^{3}H]NADH to generate a L-[4-^{3}H]homoserine sample which was mixed with U-^{14}C-labeled L-homoserine to a $^3H/^{14}C$ ratio of 5.44. The homoserine sample was then degraded to 3-hydroxypropionate by L-amino acid oxidase

Scheme VI



oxidation of the 2*S*-amino group and in situ H_2O_2-mediated decarboxylation of the α-keto acid. The $^3H/^{14}C$ ratio was raised to the anticipated 7.49. The 3-hydroxypropionate was assayed for substrate activity with liver alcohol dehydrogenase, which is known to show strict stereospecificity for *pro-R* proton removal from primary alcoholic groups,[15] but no substrate activity was detected. Esterification, to block the negative charge, to the methyl and ethyl esters was to no avail, but conversion to the benzyl ester with phenyldiazomethane yielded a pure benzyl ester after TLC. This compound showed a V_{max} of 5% that of ethanol with ADH.

Four separate incubations revealed no tritium transfer to newly formed NADH during alcohol dehydrogenase mediated oxidation, strongly indicating that tritium in the 3-hydroxypropionyl benzyl ester is in the *S* locus at C_3. To rule out the opposite possibility, that tritium is at 3*R* but there is a very large isotopic selection against removal of tritium in the ADH oxidation, we recovered the unreacted substrate molecules after 60% completion. At that point, the NADH product had no detectable tritium where 5% of the expected radioactivity could have been detected. Thus, if the sample were a (3*R*)-[3-^{3}H]-3-hydroxypropionyl benzyl ester, and $k_H/k_{3_H} > 12$, there would be a selective accumulation of tritiated substrate molecules in the unreacted pool and the $^3H/^{14}C$ ratio *would have to rise more than twofold* (since ^{14}C label monitors reaction rate of 3-protio species). In fact, the $^3H/^{14}C$ ratio of unreacted, recovered substrate molecules held constant at 7.5. Tritium is indeed in the nontransferable 3*S* locus and no isotope selection is in effect.

Thus, L-homoserine dehydrogenase catalyzed transfer of a hydride equivalent from (4*S*)-NADH to the *si* face of bound L-aspartate semialdehyde, introducing the itinerant hydrogen at the *pro-S* locus at C_4 of the L-homoserine product. Then in Table III, the sample with *upfield* C_4-*H* resonance is (4*R*)-L-[4-^{2}H]-homoserine and that with *downfield* C_4-*H* resonance is (4*S*)-L-[4-^{2}H]homoserine. With these absolute assignments in hand, we can see also from Table II that (*Z*)-[4-^{2}H]vinylglycine yields only (4*R*)-[4-^{2}H]homoserine and the (*E*)-[4-^{2}H]vinylglycine gives (4*S*)-[4-^{2}H]homoserine. Since the degradation in Scheme V involves one inversion of configuration at C_4, *the cystathionine from (Z)-[4-^{2}H]vinylglycine is a 4S-^{2}H and that from (E)-[4-^{2}H]vinylglycine is a 4R-^{2}H* as are the homocysteine and methionine species generated during degradation. Assuming a cisoid conformation of the fully conjugated key intermediate, I, as justified in the discussion section, the second half-reaction of cystathionine γ-synthase catalysis will involve attack of the thiolate atom of cosubstrate L-cysteine from below the plane at C_4 in structure I of Scheme VI.

γ-Replacement Stereochemistry. *O*-Succinyl[4-^{2}H]homoserines to [4-^{2}H]Cystathionines. The (4*R*)- and (4*S*)-L-[4-^{2}H]homoserine were chemically succinylated to the corresponding *O*-succinylhomoserines, the normal physiological substrates for cystathionine γ-synthase, and processed enzymically in the presence of L-cysteine to two samples of [4-^{2}H]cystathionines, which after purification gave NMR spectra that clearly establish that (4*R*)-*O*-succinyl-L-[4-^{2}H]homoserine (XVII) yields (4*R*)-[4-^{2}H]cystathionine, and

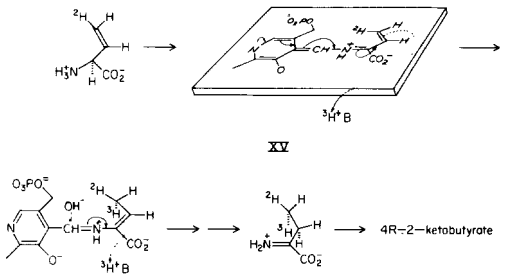
(12) Assuming chiral reprotonation of enzyme bound aminocrotonate at C_3 to yield 4*R*,3*R*, ditritioketobutyrate product as suggested in Scheme VII. We have not, in fact, determined the C_3 chirality. Degradation to acetate as outlined in the Methods section destroys this chiral, radioactive center.

(13) Hong, J. S., Brandeis University, Waltham, MA, unpublished observations.

(14) Truffa-Bachi, P.; Cohen, G. N. *Biochem. Biophys.* **1966**, *128*, 426.

(15) Alworth, W. L. In "Stereochemistry and Its Application in Biochemistry"; Wiley: New York, 1972.

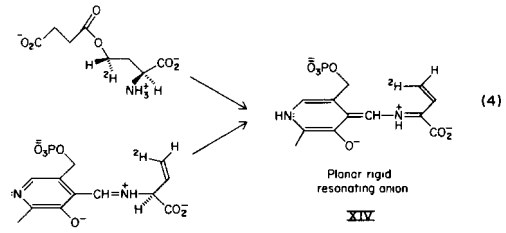
Scheme VII



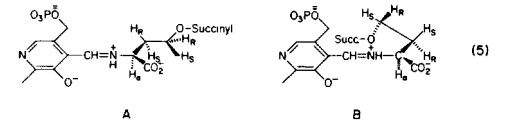
the 4*S* substrate (XVI) likewise generates (4*S*)-[4-^{2}H]cystathionine. The overall γ-replacement reaction occurs with *retention of configuration* at C_4, the γ carbon, undergoing substitution.

Discussion

Since (4*S*)-*O*-succinyl[4-^{2}H]homoserine and (*Z*)-[4-^{2}H]-vinylglycine produce the same (4*S*)-[4-^{2}H]cystathionine diastereomer, it follows that 4*S*-*O*-succinyl[4-^{2}H]homoserine yields the *Z* conformation in the intermediate vinylglycine–PLP stabilized α-carbanion. Similarly the (4*R*)-*O*-succinyl[4-^{2}H]homoserine must generate the *E* double bond isomer of the key intermediate, I.[18] (See eq 4.)



If this is so, given the result of Fuganti and colleagues[6] that the cystathionine γ-synthase removes the *pro-R* hydrogen at the β carbon preferentially, then, for example, the net β-H_R, γ-*O*-succinyl elimination to yield, from (4*S*)-*O*-succinyl[4-^{2}H]homoserine, a *Z* intermediate, XIV, must be a net *syn* process (either concerted or multiple steps). In principle, the *O*-succinylhomoserine–PLP adduct at the active site could be in two extreme rotamer distributions shown with β-H_R and γ-*O*-succinyl on the antarafacial side from H_α in the 2*S* isomer, A, or with β-H_R and γ-*O*-succinyl aligned suprafacial with regard to H_α, B (eq 5).

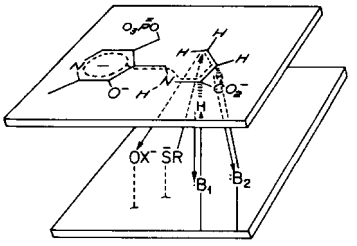


There is experimental evidence which can be interpreted strongly in favor of the rotamer B with H_α, β-H_R, and γ-*O*-succinyl all below the plane of conjugation as it develops. In particular (2*S*)-*O*-succinyl[2-^{3}H]homoserine shows 55% intramolecular tritium transfer to the β carbon during γ elimination, most economically represented as a 1,2-suprafacial $^3H^+$ transfer from a basic group disposed suprafacially.[16] Then H_α and β-H_R should be accessible from a single base, and this would represent conformer B in eq 5.

A net syn elimination of β-H_R and γ-*O*-succinyl from conformer B does yield the (*Z*)-[4-^{2}H]vinylglycine–PLP adduct, specifically as the cisoid isomer of the planar rigid resonating anion, not the transoid form, and so fixes the active-site geometry as indicated in Scheme VI. At this point, the stereochemical outcome of conversion of (*Z*)- and (*E*)-[4-^{2}H]vinylglycines to (4*R*)- and (4*S*)-[^{1}H,^{2}H,^{3}H]-2-ketobutyrates, respectively, can be interpreted. Addition of a $^3H^+$ solvent equivalent from below to the *si* face of intermediate XV will indeed generate the observed products as illustrated for the (*Z*)-[4-^{2}H]vinylglycine case, with cisoid geometry as noted in Scheme VII.

(16) Floss, H.; Schleicher, E.; Potts, R. *J. Biol. Chem.* **1976**, *251*, 5478.

Scheme VIII



Thus, the net 1,3-allylic isomerization of vinylglycine to 2-ketobutyrate catalyzed by the cystathionine γ-synthase is, in a formal sense, a 1,3-suprafacial process at the *si* face of C_2 and C_4 of the amino acid part of the amino acid–pyridoxal-P adduct. There is no internal or intramolecular 1,3-transfer of α-^{3}H of vinylglycine to the γ carbon of α-ketobutyrate, confirming the anticipated intermediacy of at least one (solvent exchangeable) enzymic active site base in the process. In fact, proton transfers to and from the α, β, and γ carbons in the γ-elimination mode, *O*-succinylhomoserine to α-ketobutyrate, are all suprafacial at the lower face of intermediate XV.[19]

In the γ-replacement mode as well, the *normal physiological reaction*, the incoming nucleophilic sulfur of cosubstrate L-cysteine also approaches only from below in the overall retention mode. Hence, all the catalytic action is on the bottom face of the substrate–coenzyme adducts as shown in Scheme VIII. It is precisely this face which is used exclusively by β-elimination and β-replacement PLP enzymes such as tryptophanase, tryptophan synthetase, *O*-acetylserine sulfhydrylase, cyanoalanine synthase, tyrosine phenol lyase, and D- and L-serine dehydrases.[17] Inclusion of the γ-carbon enzymic conversions in this list strengthens the hypothesis that a single PLP-binding domain may have evolved in an early protein in which one face, the upper of Scheme VIII, was blocked in approach by substrates, be they amino acids or protons, or for elimination of leaving groups at β or γ carbons of amino acid substrates (hence, the syn elimination sequences). Such an asymmetric binding domain appears preserved and utilized in all PLP enzymes examined for stereochemistry. How such selective shielding is arranged by the protein side chains in each instance will be a dividend of X-ray crystallographic structural analyses.

Whether cisoid intermediate geometry and overall retention is generalizable for other PLP enzymes operating at the γ carbon of amino acids remains to be determined, but the latter point at least seems the likely outcome. The availability of chiral L-[4-^{2}H]homoserines, methionines, and *E* and *Z* isomers of vinylglycine described here should facilitate such enzymic studies.

Experimental Section

I. Synthesis of [4-^{2}H]Vinylglycines. 1. 2-Hydroxy-3-[4-^{2}H]butynoic Acid (IIIa). Seven grams (0.07 mol) of 2-hydroxy-3-butynoic acid were dissolved in 10 mL of 99.8% enriched deuterated water and stirred at room temperature for 20 min. The deuterated water was distilled off. The residue was redissolved in 20 mL of deuterated water and p^{2}H was adjusted to 9.0 by 40% NaO^{2}H in ^2H_2O. The mixture was stirred at room temperature and the exchange was monitored by NMR. Deuterium exchange was stopped at 95% deuterium enrichment at C_4 position. Deuterated water was distilled off and 20 mL of fresh 99.8% deuterated water was added. The p^{2}H was readjusted to 9.0 with 40% NaO^{2}H in ^2H_2O. This procedure was repeated 3 times until the NMR showed greater than 98.5% deuterium enrichment at C_4 position. The p^{2}H of the mixture was adjusted to 1.2 with concentrated deuterium chloride and then continuously extracted with ether for 24 h. A light-brown oil weighed 6.5 g (93% yield) after removal of ether. The NMR showed a

(17) Skye, G.; Potts, R.; Floss, H. *J. Am. Chem. Soc.* **1974**, *96*, 1593.

(18) Here and elsewhere in this paper we draw the PLP structure such that the *si* face is from below, not because we have any data on this point, e.g., such as chiral BH_4^- reduction, but simply by analogy to all the other PLP–enzyme accessibility data (e.g., transaminases, β-replacement and β-elimination enzymes) which show *si* face accessibility.

(19) Schleicher, E.; Mascaro, K.; Potts, R.; Mann, D.; Floss, H. *J. Am. Chem. Soc.* **1976**, *98*, 1043.

greater than 98.5% deuterium enrichment at C_4 position.

2-Hydroxy-3-[4-^{2}H]butynoic acid (IIIa) was dissolved in ether and freshly prepared diazomethane added until the yellow color of diazomethane persisted. Ether was removed and the ester product distilled at 63 °C (1 mm). A colorless liquid of methyl 2-hydroxy-3-[4-^{2}H]butynoate soon solidified at room temperature. The product weighed 6.7 g (98% yield).

2. (*Z*)-2-Hydroxy[4-^{2}H]vinylglycolic Acid (IIIb). A 6.7-g sample (0.06 mol) of methyl 2-hydroxy-3-[4-^{2}H]butynoate was dissolved in 100 mL of acetone. To this solution were added 2 mL of freshly distilled quinoline and 300 mg of Lindlar catalyst. Hydrogenation proceeded at room temperature and 50 psi hydrogen pressure. The hydrogen uptake was stopped after 30 min, at calculated one equivalence. Catalyst was filtered off and the acetone distilled. The residue was dissolved in 50 mL of methanol and 10 g of dry Dowex 50 resin (50–100 mesh, H^+ form) was added. The mixture was stirred at room temperature for 2 h to remove quinoline. The product was distilled at 65 °C under aspirator pressure (15 mm), and 4.8 g of methyl (*Z*)-2-hydroxy-3-[4-^{2}H]butenoate was obtained (70%). Two grams of unreacted starting material was recovered.

Methyl (*Z*)-2-hydroxy-3-[4-^{2}H]butenoate was saponified with 1M LiOH at room temperature to give (*Z*)-2-hydroxy-3-[4-^{2}H]vinylglycolate (IIIb) (4.3 g, 95% yield).

3. (*Z*)-DL-[4-^{2}H]Vinylglycine (III). Four grams (0.03 mol) of IIIb was dissolved in 20 mL of anhydrous ether. The mixture was cooled to 0 °C in an ice bath, and 2.6 mL of PBr_3 in 10 mL of anhydrous ether was added over a period of 20 min, and then the reaction was stirred in the dark at room temperature for 20 h. The reaction mixture was then cooled to 0 °C and 15 mL of water was added cautiously with vigorous stirring. The mixture was extracted 3 times with 20-mL portions of ether. The combined ether extracts were dried over anhydrous magnesium sulfate, and the ether was removed to give a yellow oil of (*Z*)-2-bromo-[4-^{2}H]vinylglycolate (5.4 g, 80%). This compound was cooled to 0 °C, and 200 mL of 20% $(NH_4)_2CO_3/NH_4OH$ solution was added. The mixture was stirred vigorously for 24 h at 4 °C and then at room temperature for an additional 48 h. The aqueous ammonia was removed in vacuo and the semisolid residue was redissolved in 10 mL of water. The mixture was passed through 100 g of Dowex 50 cation-exchange column (50–100 mesh, H^+ form), and the column was washed with water until the elute was neutral. It was then eluted with 1 N NH_4OH; ninhydrin-positive fractions were combined. An off-white solid was obtained upon removal of aqueous ammonia and 400 mg of crystalline (*Z*)-DL-[4-^{2}H]vinylglycine (III) was obtained after repeated recrystallizations from aqueous ethanol (20% yield): mp 234 °C decomp; NMR 4.30 (1 H, d, J = 8.0 Hz), 5.51 (1 H, d, J = 11.0 Hz), 6.07 (1 H, m).

4. (*E*)-DL-[3,4-$^2H_2$]Vinylglycine (IV). Methyl 2-hydroxy-3-butynoate (5.7 g, 0.05 mol) was hydrogenated with deuterium gas by the identical procedure described above for compound IIIb. Subsequent saponification gave 2.3 g of (*E*)-[3,4-$^2H_2$]vinylglycolate (IVa) (50% yield). Deuterium enrichment at C_4 *E* position is ≥96% by NMR analysis.

(*E*)-DL-[3,4-$^2H_2$]Vinylglycine (IV) was obtained by the same procedure described above for compound III: 200 mg of white crystalline material was obtained; mp 235 °C decomp; NMR 4.30 (1 H, br s), 5.47 (1 H, br s).

II. Synthesis of (4*R*)- and (4*S*)-*O*-Succinyl-L-[4-^{2}H]homoserines (XII and XIII). 5. (4*S*)-L-[4-^{2}H]-Homoserine (XIII). L-Aspartate semialdehyde (11 mg, 0.1 mmol) and 0.11 mmol of (4*S*)-[4-^{2}H]NADH[20] were added to 20 mL of potassium phosphate buffer (0.1 M, pH 6.6). Five units [5 μmol min^{-1} (mg of proten)$^{-1}$] of L-homoserine dehydrogenase was added at room temperature. L-Homoserine formation was monitored by the disappearance of NADH absorbance at 340 nm. After 1 h, there was less than 5% of NADH left. The mixture was passed through 5 mL of Dowex 50 cation-exchange column (50–100 mesh, H^+ form) and washed with 25 mL of water. The column was then eluted with 1 N NH_4OH and the mixture of amino acids was further purified by HPLC (Whatman Partisel PXS 10/25 SCX column). A 5.1-mg amount of (4*S*)-L-[4-^{2}H]homoserine (XIII) was obtained; 270-MHz NMR showed a chemical shift of 1029.3 Hz and integrated for one hydrogen.

6. (4*R*)-L-[4-^{2}H]Homoserine (XII). L-Propargylglycine (113 mg, 1 mmol) was partially deuterated with deuterium gas and Adams catalyst in deuterated water. The reaction was stopped at the consumption of 1 equiv of deuterium gas. The 4,5-dideuterio-L-allylglycine was purified by HPLC and recrystallization (55 mg, 49% yield). Ozonolysis of this compound in 1 N $^2HCl/^2H_2O$ yielded L-[4-^{2}H]aspartate semialdehyde. Incubation of 11 mg (0.1 mmol) of L-[4-^{2}H]aspartate semialdehyde with 0.11 mmol of NADH and 5 units of L-homoserine dehydrogenase resulted, after HPLC purification, in 5.3 mg of (4*R*)-L-[4-^{2}H]homoserine (XII).

7. (4*R*)- and (4*S*)-*O*-Succinyl-L-[4-^{2}H]Homoserines (XVI and XVII). (4*R*)-L-[4-^{2}H]homoserine (XII) (5.0 mg, 0.045 mmol) and sodium bicarbonate (10 mg) were dissolved in 0.5 mL of water. The mixture was stirred at room temperature and 10 μL of carbobenzoxy chloride was added, and the mixture was stirred for 3 h. The reaction mixture was washed twice with ether. The aqueous phase was acidified to pH 1 with concentrated HCl and extracted 3 times with 5-mL portions of ether. The semisolid *N*-(carbobenzoxy)-L-homoserine obtained upon removal of ether (9.5 mg) was dissolved in 1 mL of pyridine, 5 mg of succinyl anhydride were added, and mixture was refluxed for 3 h. The pyridine was removed in vacuo. The residue was redissolved in 1 mL of water and was washed twice with either. The aqueous phase was then acidified to pH 1 with 6 N HCl and extracted 3 times with ether. Upon removal of ether, 15 mg of yellowish solid of *O*-succinyl-*N*-(carbobenzoxy)-L-homoserine was obtained. This compound can be decolorized by heating briefly with charcoal. The product weighed 14.3 mg (94% yield). *O*-Succinyl-*N*-(carbobenzoxy)-L-homoserine was dissolved in 1 mL of glacial acetic acid, and 3 mg of 10% Pd/C was added. Hydrogenation proceeded under atmospheric pressure of hydrogen and room temperature until one equivalence of hydrogen was consumed. Acetic acid was distilled off under reduced pressure, and the residual white solid was recrystallized from hot water. Seven milligrams of *O*-succinyl-L-homoserine (XVI) was obtained (95% yield).

III. Degradation of L-[γ-^{2}H]Cystathionines (V and VI). 8. (4*R*)-L-[4-^{2}H]Homoserine (XII). 4*S*-L-[4-^{2}H]Cystathionine (V) (54 mg, 0.25 mmol) was suspended in 50 mL of 0.1 M potassium phosphate buffer (pH 7.5, 1 mM Mg^{2+}, 1 mM Fe^{3+}) and 1 unit of β-cystathionase [1 μmol min^{-1} (mg of protein)$^{-1}$] was added. The mixture was incubated at room temperature for 24 h with a thin stream of oxygen bubbled into the mixture. The reaction mixture was passed through 100 mL of Dowex 50 cation-exchange column (200–400 mesh, H^+ form), and the column was washed with 100 mL of water. It was then eluted with 2 N NH_4OH, and the ninhydrin positive fractions were combined. An off-white solid was obtained upon removal of solvent. After recrystallization from hot water, 19.1 mg of (4*S*)-L-[4-^{2}H]homocystine was obtained (58% yield).

9. (4*S*)-L-[4-^{2}H;*methyl*-$^2H_3$]methionine (VIII). (4*S*)-L-[4-^{2}H]-Homocystine (19.1 mg) was dissolved in 15 mL of liquid ammonia at −50 °C under an argon atmosphere. Small pieces of sodium metal were added until a blue color persisted for at least 5 min. Dry trideuteriomethyl iodide (50 μL) was added, and the dark-blue color faded immediately. An additional 300 μL of trideuteriomethyl iodide was added, and the reaction flask was allowed to warm up to room temperature. Ammonia was evaporated spontaneously to leave a large amount of white solid. This was dissolved in 5 mL of water and the pH was adjusted to 3.0 with 10% HI solution. The product methionine was purified by passing through a Dowex 50 cation-exchange column and was crystallized to yield 9.8 mg of crystalline (4*S*)-L-[4-^{2}H]methionine (VIII) (45% yield).

10. (4*R*)-L-[4-^{2}H]Homoserine γ-Lactone (XI). (4*S*)-L-[4-^{2}H]-Methionine (VIII) (9.8 mg) and 30 mg of iodoacetic acid was dissolved in 1 mL of water. The mixture was kept at 40 °C in the dark for 24 h. The aqueous mixture was washed 3 times with a 1-mL portion of ethyl acetate, and the pH was adjusted to 3.0 with 0.1 N NaOH. Solvent was removed in vacuo. One milliliter of dimethylformamide was added to the residue, and the resulting mixture was heated to 100 °C for 2 h. The mixture was cooled to room temperature and dimethylformamide was distilled off. The product (4*R*)-L-[4-^{2}H]homoserine γ-lactone (XI) (4.9 mg) was purified by Dowex 50 cation-exchange column (70% yield from methionine).

11. (4*R*)-L-[4-^{2}H]Homoserine (XII). The lactone XI was heated with 1 mL of 1 N NaOH for 1 h at 100 °C. The product homoserine XII was purified by the procedure described above.

Enzyme Preparations and Assays. Cystathionine γ-Synthase. This enzyme, isolated from *Salmonella typhimurium*, was purified to homogeneity according to the procedure by Kaplan and Flavin.[21] The turnover number for cystathionine formation is 2.5×10^3 mmol min^{-1} (mmol of enzyme)$^{-1}$. In the absence of cysteine, the enzyme carries out γ elimination at 5.0×10^2 mmol min^{-1} (mmol of enzyme).

Standard Assay Condition. One milliliter of aqueous buffer mixture containing 100 mM potassium phosphate (pH 8.4), 25 mM *O*-succinyl-L-homoserine, 30 mM NADH, and 0.1 mg of lactic acid dehydrogenase was incubated at 37 °C. Cystathionine γ-synthase (0.01 mg) was added at time zero, and the disappearance of NADH absorbance at 340 nm was monitored.

Homoserine Dehydrogenase. Isolated from *E. coli*, this enzyme was purified to homogeneity in Dr. G. N. Cohen's laboratory according to

(20) Oppenheimer, N. J.; Arnold, L. J.; Kaplan, N. O. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 3200.

(21) Kaplan, M.; Flavin, M. *J. Biol. Chem.* **1966**, *241*, 5781.

the published procedure.[14] Turnover number for the homoserine formation is 45 μmol min^{-1} (mg of enzyme)$^{-1}$.

Standard Assay Procedure. One milliliter of aqueous buffer mixture containing 5 mM L-aspartate semialdehyde, 10 mM NADH, and 100 mM potassium phosphate, at pH 6.6, was incubated at room temperature. Reaction was initiated by addition of 0.1 unit [μmol min^{-1} (mg of enzyme)$^{-1}$] of enzyme. L-Homoserine formation was monitored as disappearance of NADH absorbance at 340 nm.

β-Cystathionase. Isolated from *E. coli*, this enzyme was purified to homogeneity by Dr. J. S. Hong of Brandeis University.[13] The turnover number for L-cysteine formation is about 50 μmol min^{-1} (mg of enzyme)$^{-1}$.

Standard Assay Condition. One milliliter of aqueous buffer mixture containing 160 mM potassium phosphate (pH 7.4), 1 mM $MgSO_4$, 2 mL L-cystathionine, and 0.1 mg of Ellman's reagent was incubated at 37 °C. Enzyme β-cystathionase was added at time zero, and the disappearance of chromophore at 412 was monitored.

For NMR studies, all the deuterated amino acid samples were dissolved in 99.8% ^2H_2O (p^{2}H 7.2). NMR spectra were obtained on Dr. A. Redfield's 270-MHz NMR spectrometer (Brandeis University). Temperature was fixed at 29.6 °C, and dioxane was used as internal standard.

Acknowledgment. We are indebted to Professor G. N. Cohen for his generous gift of homogeneous aspartate kinase/homoserine dehydrogenase from *E. coli* and to Professor J. S. Hong for his sample of β-cystathionase. Thanks to Professor A. Redfield for his assistance and the use of his 270-MHz NMR. This research was supported in part by Grant GM 20011 from NIH.

Temperature-Dependent Spin-State Equilibrium in Aquo and Hydroxo Ferric Heme Octapeptide Complexes. Model Systems for the Spin Equilibrium of Ferric Hemoproteins

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Abstract: The temperature dependencies of the magnetic susceptibilities of aquo and hydroxo ferric heme octapeptide complexes were investigated as models for the temperature-dependent spin-state equilibria of ferric hemoproteins. The shifts in proton NMR signal of the sodium 4,4-dimethyl-4-silapentanesulfonate (DSS) caused by the heme octapeptide were measured from below room temperature to well above room temperature in aqueous and aqueous–ethylene glycol solutions. Magnetic susceptibilities calculated from the shifts do not exhibit simple Curie behavior as would be expected for pure high-spin or low-spin systems. The temperature dependencies of the susceptibilities are consistent with thermal spin-state equilibria: high spin ($S = {}^5/_2$) ⇄ low spin ($S = {}^1/_2$). The equilibrium constants at 25 °C are 2.37 for aquoheme octapeptide in aqueous solution, 2.04 for (aquo/ethylene glycol)heme octapeptide in D_2O–ethylene glycol solution, 17.5 for hydroxoheme octapeptide in aqueous solution, and 1.79 for (hydroxo/alkoxo)heme octapeptide in D_2O–ethylene glycol solution. Thermodynamic values determined from plots of ln k vs. $1/T$ are $\Delta H° = -9400 \pm 400$ J/mol and $\Delta S° = -24.4 \pm 1.3$ J/(mol·K) for aquoheme octapeptide in aqueous solution; $\Delta H° = -8700 \pm 200$ J/mol and $\Delta S° = -23.4 \pm 0.7$ J/(mol·K) for aquo/ethylene glycol ferric heme octapeptide in 50% (v/v) ethylene glycol–D_2O solution; $\Delta H° = -35\,000 \pm 200$ J/mol and $\Delta S° -94 \pm 6$ J/(mol·K) for hydroxo ferric heme octapeptide in aqueous solution; $\Delta H° = -15\,900 \pm 1200$ J/mol and $\Delta S° = -48.5 \pm 3.8$ J/(mol·K) for hydroxo/alkoxo ferric heme octapeptide in 50% (v/v) ethylene glycol–D_2O solution. The thermodynamic data for these model systems are compared with those of hemoproteins. The results indicate that while suitable axial ligands to the heme iron give rise to spin-state equilibria in model systems, the thermodynamic values for hemoproteins are determined by interactions of the coordination center with the protein.

A number or reports have described model systems for the active sites of hemoproteins.[1-8] A comparison of the properties of such models to those of the proteins can be used to determine the effect that protein structure has on the intrnisic properties of the coordination center. Several studies[9-24] have described the thermal spin-state equilibria of many ferric hemoproteins and their ligand substitution derivatives in an attempt to provide information relevant to their fucntion in biological systems. Many of these proteins appear to be characterized by coordination of the heme iron to an imidazole group of a histidyl residue at the fifth coordination position and the H_2O (OH^-) or another amino acid side chain at the sixth coordination position. Substitution of the ligand at the sixth coordination position by added ligands is ac-

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