Registry No. Citrate lyase, 9012-83-3; citric acid, 77-92-9; (−)-*erythro*-2-fluorocitric acid, 31654-44-1; (+)-*erythro*-2-fluorocitric acid, 74841-44-4; (−)-*erythro*-2-hydroxycitric acid, 6205-15-8; (+)-*erythro*-2-hydroxycitric acid, 27750-11-4; (−)-*threo*-2-hydroxycitric acid, 27750-10-3; (+)-*threo*-2-hydroxycitric acid, 6385-10-0.

References

Bergmeyer, H., Ed. (1974) *Methods of Enzymatic Analysis*, p 442, Academic Press, New York.

Bowen, T. J., & Mortimer, M. G. (1971) *Eur. J. Biochem. 23*, 262.

Buckel, W., Buschmeier, V., & Eggerer, H. (1971) *Hoppe-Seyler's Z. Physiol. Chem. 352*, 1195.

Carrell, H. L., Glusker, J. P., Villafranca, J. J., Mildvan, A. S., Dummel, R. J., & Kun, E. (1970) *Science (Washington, D.C.) 170*, 1412.

Dimroth, A., & Eggerer, H. (1975a) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3458.

Dimroth, A., & Eggerer, H. (1975b) *Eur. J. Biochem. 53*, 227.

Dimroth, A., Loyal, R., & Eggerer, H. (1977) *Eur. J. Biochem. 80*, 479.

Dummel, R. J., & Kun, E. (1969) *J. Biol. Chem. 244*, 2966.

Fanshier, D. W., Gottwald, L. K., & Kun, E. (1964) *J. Biol. Chem. 239*, 425.

Filler, R., Ed. (1976) *ACS Symp. Ser. No. 28.*

Glusker, J. P. (1971) *Enzymes, 3rd Ed. 5*, 413.

Glusker, J. P. (1980) *Acc. Chem. Res. 13*, 345.

Glusker, J. P., & Srere, P. A. (1973) *Bioorg. Chem. 2*, 301.

Goldstein, J. A., Cheng, Y., Marletta, M. A., & Walsh, C. (1978) *Biochemistry 17*, 5567.

Lewis, Y. S., & Neelakantan, S. (1965) *Phytochemistry 4*, 619.

Marletta, M. A., Srere, P. A., & Walsh, C. T. (1981) *Biochemistry 20*, 3719.

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

Peters, R. (1972) *Carbon-Fluorine Compounds: Chemistry, Biochemistry and Biological Activities, a Ciba Foundation Symposium*, London, September 13–15, 1971, Associated Scientific Publishers, Amsterdam.

Rokita, S. E., Srere, P. A., & Walsh, C. T. (1982) *Biochemistry 21*, 3765.

Singh, M., & Srere, P. A. (1971) *J. Biol. Chem. 246*, 3847.

Singh, M., Dempsey, W. B., & Srere, P. A. (1975) *J. Bacteriol. 124*, 686.

Sivaraman, H., & Sivaraman, C. (1979) *FEBS Lett. 105*, 267.

Srere, P. A. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 57.

Srere, P. A., Bottger, B., & Brooks, G. C. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1201.

Stallings, W. C., Blount, J. F., Srere, P. A., & Glusker, J. P. (1979) *Arch. Biochem. Biophys. 193*, 431.

Stallings, W. C., Monti, C. T., Belvedere, J. F., Preston, R. K., & Glusker, J. P. (1980) *Arch. Biochem. Biophys. 203*, 65.

Streitwieser, A., & Heathcock, C. H. (1976) *Introduction to Organic Chemistry*, p 429, MacMillan, New York.

Sullivan, A., Singh, M., Srere, P. A., & Glusker, J. P. (1977a) *J. Biol. Chem. 252*, 7583.

Sullivan, A., Triscari, J., & Spiegel, H. E. (1977b) *Am. J. Clin. Nutr. 30*, 777.

Walsh, C. T. (1983) *Adv. Enzymol. Relat. Areas Mol. Biol.* (in press).

Chiral Instability at Sulfur of *S*-Adenosylmethionine†

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ABSTRACT: *S*-Adenosylmethionine, generated enzymically in chirally pure form (*S* configuration at sulfur), undergoes simultaneous irreversible conversion to 5′-deoxy-5′-(methylthio)adenosine and homoserine with a rate constant of 6×10^{-6} s^{-1} at pH 7.5 and 37 °C and reversible conversion to an enzymically inactive stereoisomer (*R* configuration at sulfur) with a forward rate constant of 8×10^{-6} s^{-1} at pH 7.5 and 37 °C. These forms of instability require small turnover times and/or stabilization through macromolecular binding for *S*-adenosylmethionine, if organisms that utilize it are to avoid losses of metabolic energy.

The compound *S*-adenosylmethionine (AdoMet[1]), the methyl donor for numerous enzymic transmethylation reactions which play the most diverse roles in biochemistry (Cantoni, 1952, 1953, 1960; Usdin et al., 1979, 1981), is structurally complex. This molecular complexity is thought to be critical for enzyme–substrate interactions in the catalytic transition state, which lead to the large catalytic accelerations that transmethylases achieve [10^{16} for catechol *O*-methyltransferase (COMT); Olsen et al., 1979]. On the other hand, the elaborate structure is paid for by the organism through the very high energy cost of AdoMet biosynthesis (Cantoni, 1952, 1960). Atkinson (1977) writes "... in being reduced and activated to form the methyl group of *S*-adenosylmethionine, a carbon atom from glucose is promoted from an average value of 6.3 [ATP] equivalents (38/6) to a cost of 12 equivalents. Active methyl is probably the most expensive metabolic compound or group on a per-carbon basis". This suggests that organisms should experience a strong evolutionary pressure toward preservation and efficient utilization of AdoMet.

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[1] Abbreviations: AdoHcy, *S*-adenosyl-L-homocysteine; AdoMet, *S*-adenosylmethionine; AdoMet synthetase, *S*-adenosylmethionine synthetase; ATP, adenosine 5′-triphosphate; COMT, catechol *O*-methyltransferase; DHA, 3,4-dihydroxyacetophenone; DTT, dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; InoHcy, *S*-inosyl-L-homocysteine; MTA, 5′-deoxy-5′-(methylthio)adenosine; SA, specific activity; HPLC, high-pressure liquid chromatography.

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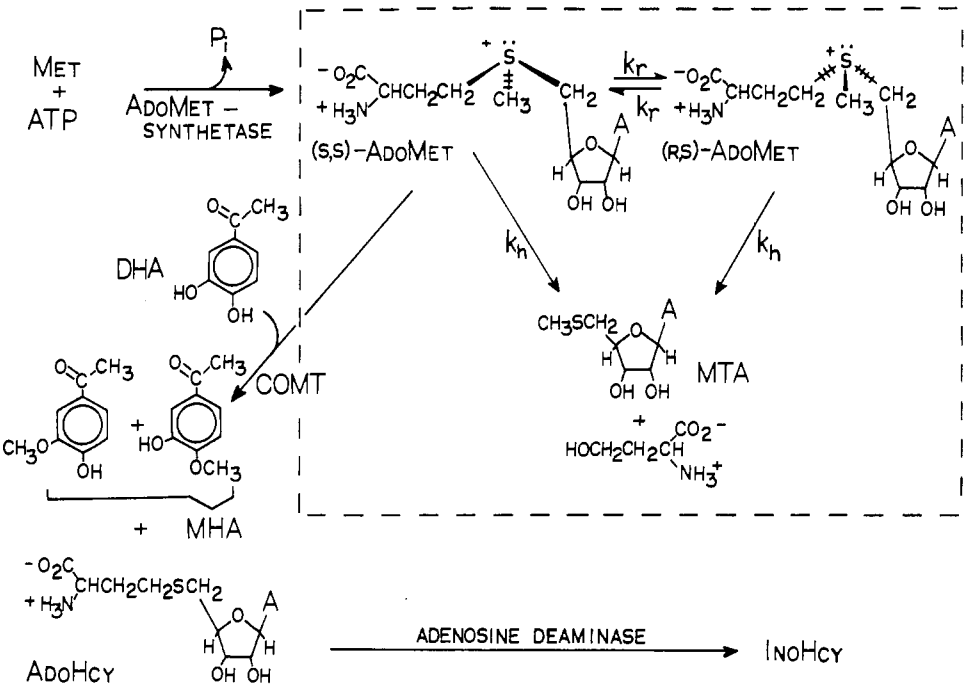
Scheme I



Such efficiency is made more difficult to achieve by several types of intrinsic instability in AdoMet, of which the enzymically active stereoisomer (De la Haba et al., 1959; Cornforth et al., 1977) is 5′-[[(3*S*)-3-amino-3-carboxypropyl]methyl-(*S*)-sulfonio]-5′-deoxyadenosine. This stereoisomer has been known as (−)-AdoMet but we shall refer to it as (*S*,*S*)-AdoMet. The molecule can, for example, fragment by loss of adenine (Cantoni, 1953; Baddiley et al., 1953; Parks & Schlenk, 1958; Borchardt, 1979) or hydrolyze by a mechanistically complicated route to 5′-deoxy-5′-(methylthio)-adenosine (MTA) and homoserine (Parks & Schlenk, 1958; Mather, 1958; Mudd, 1959; Zappia et al., 1977, 1979).

Furthermore, it seemed likely to us that the *S* chirality at the sulfonium pole might be lost through spontaneous racemization. This was suggested both by kinetic studies of racemization of simpler sulfonium salts (Darwish & Tourigny, 1966; Scartazzini & Mislow, 1967; Darwish, 1968; Garbesi et al., 1970; Darwish & Scott, 1973) and by reports (Stolowitz & Minch, 1981), confirmed by us, that commercial samples of AdoMet from biological sources can contain around 20% of the enzymically inactive (*R*,*S*)-AdoMet.

In this paper, we report a quantitative study of the loss of chirality at the sulfonium pole of AdoMet. The process, as will be seen, competes with nonenzymic hydrolysis to MTA. We also consider briefly the biological implications of the various modes of AdoMet instability.

Materials and Methods

Chemicals. L-[*methyl*-^{3}H]Methionine (SA 79 Ci/mmol) was purchased from Amersham. Adenosine 5′-triphosphate (ATP), D-amino-acid oxidase, dithiothreitol (DTT), Hepes buffer, MTA, and L-methionine were purchased from Sigma Chemical Co.

Biosynthetic System. AdoMet was synthesized enzymically with AdoMet synthetase (ATP:L-methionine *S*-adenosyltransferase, EC 2.5.1.6) isolated from Sprague-Dawley rat livers with a SA of 210 pmol of AdoMet formed (mg of protein)$^{-1}$ min^{-1} (Kunz et al., 1980). A typical reaction solution consisted of 31.75 mM Hepes buffer (pH 7.5), 3.17 mM DTT, 9.52 mM $MgCl_2$, 95.2 mM KCl, 12% (v/v) glycerol, 1.27 mM ATP, 15.8 μM L-[*methyl*-^{3}H]methionine (SA 500 mCi/mmol) and 340 μg of AdoMet synthetase [SA 210 pmol of AdoMet formed (mg of protein)$^{-1}$ min^{-1}]. The reaction solution was incubated at 37 °C for 24 h while the progress of the reaction was monitored by subjecting aliquots of the solution to the separation and assay described in the analytical section below. The reaction was complete after 6 h with 45.4% of the starting methionine remaining apparently unreacted. However, all or nearly all of this residue had been oxidized to the sulfoxide, which cochromatographs with methionine (Savige & Fontana, 1977).

Analytical System. The separation of radioactive methionine, AdoMet, MTA, and methylated products (structures shown in Scheme I) was accomplished on a Perkin-Elmer Series III HPLC equipped with a Du Pont Zorbax ODS (4.6 mm × 25 cm) C-18 reversed-phase column. The program in terms of the percentage of solvent A was designed as follows: 0.5–30%, 30–50%, and 50–80% for 18, 10, and 10 min, respectively. Solvent A consisted of acetonitrile and (pH 3.3) phosphate buffer (75%:25%), and solvent B consisted of phosphate buffer (pH 7.0). The retention times in this system for methionine, AdoMet, MTA, and methylated products were 4, 14, 20, and 30 min, respectively. Therefore, the eluent was collected from 3–8, 11–17, 18–24, and 26–32 min, correspondingly, and the total radioactivities were quantitated by liquid scintillation spectrometry (Beckman LS-7500).

Rate of Racemization of Sulfonium Center. An enzymic resolution method (De la Haba et al., 1959; Borchardt & Wu, 1976) was used to follow the racemization of the AdoMet sulfonium center. The AdoMet biosynthetic system described above was prepared and incubated at 37 °C. Two aliquots were withdrawn from the mixture every 2 h and were subjected to the separation described in the analytical section. One aliquot (200 μL) was analyzed for the total AdoMet produced in the biosynthetic reaction as a control, and the second aliquot (200 μL) was added to a solution (300 μL) containing 833 μM 3,4-dihydroxyacetophenone (DHA), 284 μg of COMT [SA 12.1 nmol of product formed (mg of protein)$^{-1}$ min^{-1}], and 30.8 μg of adenosine deaminase [SA 1.38 nmol of product formed (mg of protein)$^{-1}$ min^{-1}] isolated from Takadiastase (Sharpless

& Wolfenden, 1967), which could convert the product inhibitor *S*-adenosyl-L-homocysteine (AdoHcy) to *S*-inosyl-L-homocysteine (InoHcy) (Coward & Wu, 1973). COMT methylates DHA by using only (*S,S*)-AdoMet of the mixture of AdoMet diastereomers. Reactions were carried out at 37 °C for 10 min. The amount of COMT used was sufficient to consume 10 nmol of AdoMet (21.2 μM) during 10-min incubation time. After the COMT reaction was complete, the solution was separated and analyzed by HPLC as described above. The amount of methylated products represents the amount of (*S,S*)-AdoMet, and the remaining AdoMet must then be (*R,S*)-AdoMet.

Demonstration of Loss of AdoMet Chirality at Sulfonium Center. [*methyl*-^{3}H]AdoMet (17 nmol) was prepared in the biosynthetic system as described above and further purified with a cationic SP-Sephadex column (Glazer & Peale, 1978). Twenty percent of the radioactivity (a) remained unreacted in the transmethylation reaction catalyzed by COMT and (b) cochromatographed with AdoMet on HPLC. This suggested the presence of some unreactive stereoisomer of AdoMet, with either the D configuration at the methionine head or the *R* configuration at sulfur. The optical purity at the α-carbon of methionine was first examined by a D-amino-acid oxidase assay (Yagi, 1971). Neither the commercial, radioactively labeled methionine nor the acid hydrolysate of the biologically inactive AdoMet stereoisomer (Schlenk & Zydek-Cwick, 1969) indicated any possible contamination by D-methionine, when subjected to the D-amino-acid oxidase assay. Since the L-methionine head of AdoMet is chirally intact, loss of chirality has occurred at the sulfonium pole.

Results and Discussion

Scheme I summarizes the system as used in this study. The synthesis of chirally pure (*S,S*)-AdoMet is accomplished on a relatively short time scale, and the solution is then thermostated. HPLC analyses were conducted shortly after initiation of the synthesis (t = 0 h) and then at 2, 4, and 6 h and a number of subsequent times. At t = 0, the methionine–methionine sulfoxide peak accounted for a large fraction (0.94) of total radioactivity, but this had decreased to 0.49 at t = 2 h and to 0.45 at t = 4 h and was constant at about 0.43–0.44 at t = 6 h and subsequently. The fraction of radioactivity present in the other peaks changed in accord with the chemistry shown within the dashed box of Scheme I.

Two processes go forward simultaneously: racemization at the sulfonium pole of AdoMet and hydrolytic formation of MTA. We have assumed (a) that the forward (*S,S* → *R,S*) and reverse (*R,S* → *S,S*) racemization rate constants are equal, although this need not be so for this diastereomer interconversion, (b) that the rate constants for hydrolysis to MTA and homoserine of (*S,S*)-AdoMet and (*R,S*)-AdoMet are equal, and (c) that the amount of (*R,S*)-AdoMet present at t = 0 can be neglected. All assumptions are justified by the data, as will be shown below.

The kinetic law for the system within the dashed box, with these assumptions, is given by eq 1–3.

$$2[(S,S)\text{-AdoMet}]_t = [(S,S)\text{-AdoMet}]_0(e^{-k_h t} + e^{-(k_h+2k_r)t}) \quad (1)$$

$$2[(R,S)\text{-AdoMet}]_t = [(S,S)\text{-AdoMet}]_0(e^{-k_h t} - e^{-(k_h+2k_r)t}) \quad (2)$$

$$[\text{MTA}]_t = [(S,S)\text{-AdoMet}]_0(1 - e^{-k_h t}) \quad (3)$$

The three variable concentrations of eq 1–3 can be determined experimentally as follows (see above for details). (1) Total [AdoMet] and $[\text{MTA}]_t$ can be measured by subjecting a sample of the reaction solution, collected at t, to HPLC analysis. (2) Since COMT is specific for (*S,S*)-AdoMet, the latter can be determined by subjecting an aliquot of reaction solution, collected at time t, to COMT treatment. If the COMT concentration is large, methylation of the methyl acceptor (here DHA) will be faster than racemization or hydrolysis and will produce an amount of methylated product, MHA, equal to $[(S,S)\text{-AdoMet}]_t$. (3) $[(R,S)\text{-AdoMet}]_t$ is calculated as the difference in $[\text{AdoMet}]_t$ and $[(S,S)\text{-AdoMet}]_t$.

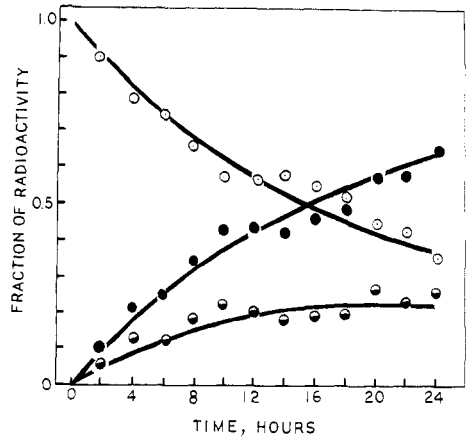


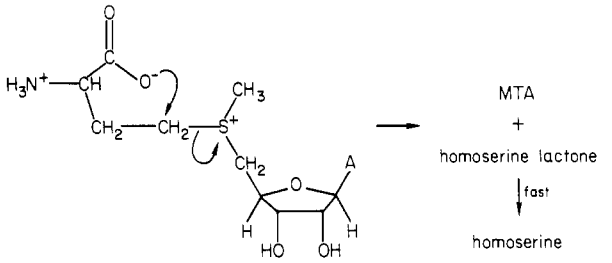
FIGURE 1: Time course concentrations of (*S,S*)-AdoMet [curve 1 (⊙)], (*R,S*)-AdoMet [curve 2 (⊖)], and, for clarity, the sum of (*R,S*)-AdoMet and MTA [curve 3 (●)] in a 24-h period following initiation of the biosynthesis of (*S,S*)-AdoMet. The curves were calculated from eq 1–3 of the text with $k_r = 8 \times 10^{-6}$ s^{-1} and $k_h = 6 \times 10^{-6}$ s^{-1}.

Figure 1 shows a plot of these three concentrations over a time period of 24 h. The concentrations are expressed as a fraction of the total radioactivity consumed in the synthetic reaction. The curves shown were calculated from eq 1–3 with $k_r = 8 \times 10^{-6}$ s^{-1} and $k_h = 6 \times 10^{-6}$ s^{-1}. The values of the rate constants were obtained by nonlinear least-squares fitting of the more general kinetic laws derived without the use of assumptions a and c above; assumption b was employed in the fitting procedure. The agreement of the curves in Figure 1 with the data shows that all three assumptions are justified. Around 24 h and slightly earlier, the ratio of (*S,S*)-AdoMet to (*R,S*)-AdoMet (points about curves 1 and 2) seems to be approaching unity: this should be true if assumptions a and b are true, since at 24 h racemization has proceeded to 75% completion and hydrolysis to 40% completion. Furthermore, the points about curve 1 extrapolate to unity and the points about curve 2 to 0 at t = 0; this confirms assumption c.

The racemization rate constant of $k_r = 8 \times 10^{-6}$ s^{-1} for AdoMet at 37 °C in water can be compared with values of 6.5×10^{-6} s^{-1} (50 °C, methanol) for (methylethylbenzyl)-sulfonium perchlorate (Darwish, 1968) and 8.6×10^{-4} s^{-1} (50 °C, acetic acid) for (methylethyladamantyl)sulfonium perchlorate (Scartazzini & Mislow, 1967). Neither of these compounds is a good model for AdoMet, and the temperatures were higher than 37 °C; but, no good model seems to have been studied at 37 °C. Effectively, AdoMet racemizes at a rate intermediate between the rates for the other two compounds, since the assumption of any activation energy between the reasonable limits of 10 and 50 kcal mol^{-1} will lead to a rate constant at 37 °C that is smaller than 8×10^{-6} s^{-1} for the methylethylbenzyl compound and larger than 8×10^{-6} s^{-1} for the methylethyladamantyl compound.

The decomposition of AdoMet to MTA has been studied in several laboratories (Parks & Schlenk, 1958; Mather, 1958; Mudd, 1959; Zappia et al., 1977, 1979) and was shown to

Scheme II



depend on the presence of the carboxyl group. It is logically supposed to proceed by intramolecular cyclization, followed by hydrolysis of the lactone (Scheme II). The rate constant found by us, $k_h = 6 \times 10^{-6}$ s^{-1} at 37 °C, is consistent with that estimated from the data of Zappia et al. ($k_h = 4 \times 10^{-3}$ s^{-1} at 100 °S) if the enthalpy of activation is 23–24 kcal mol^{-1}. This value is quite reasonable, Swain & Taylor (1962) having found 28.5 kcal mol^{-1} for reaction of phenoxide ion with trimethylsulfonium ion. The cyclization mechanism is further supported by a comparison of the rate constant with that expected for a similar intermolecular reaction. Extrapolation of Swain and Taylor's rate constant for phenoxide and trimethylsulfonium ion to 37 °C, correction by a statistical factor of 3, division by 30 to represent displacement at a primary rather than methyl center (Streitweiser, 1962), and correction from phenoxide to carboxylate nucleophilicity by use of Coward and Sweet's (1971) β value of 0.3 yields an estimated intermolecular rate constant of about 10^{-12} M^{-1} s^{-1}. When compared to the observed 6×10^{-6} s^{-1}, this produces an "intramolecular molarity" of about 10^6 M. This is just what is observed by Coward et al. (1976) for similar intramolecular reactions of sulfonium compounds.

The biological significance of these results lies in the waste of biosynthetic energy to which an organism would be exposed if the expensive (*S*,*S*)-AdoMet were converted to an enzymically unusable form in vivo. With a half-life time of around 12 h, under the physiological conditions simulated in our experiments, (*S*,*S*)-AdoMet decays to a 50:50 mixture with its enzymically inactive diastereomer. Under the same conditions, (*S*,*S*)-AdoMet is irreversibly lost through MTA formation with a half-life time of about 32 h.

Considering the 12 ATP synthetic cost of (*S*,*S*)-AdoMet, we can calculate that within a 4-h period, 1 ATP equiv of (*S*,*S*)-AdoMet will be lost through irreversible decomposition to MTA. This places a fundamental constraint on the molecular physiology of all AdoMet-utilizing organisms: either all AdoMet must be turned over within a time short compared to 4 h or some binding entity capable of chemical stabilization of AdoMet must be present. Alternatively, other considerations might outweigh the energy loss, which would then be tolerated. Note that the constraint on turnover time applies to all individual pools of AdoMet where it exists in homogeneous solution not just to the aggregate of AdoMet in the organism, an organ, or a tissue. Several recent papers may be significant in regard to these considerations. First, the half-life time of AdoMet methyl groups has been determined as 3.5–9 min in human liver (Mudd et al., 1980), 10 min in rat liver (Baldessarini, 1975), and 20–25 min in perfused rat liver (Duerre, 1981). Second, the existence of an AdoMet-binding protein has been reported (Smith, 1976).

A similar calculation indicates that 1 ATP equiv of (*S*,*S*)-AdoMet would be converted to (*R*,*S*)-AdoMet in 3 h. This does not constitute a permanent loss, however: as the (*S*,*S*)-AdoMet is consumed enzymically, the (*R*,*S*)-AdoMet will revert to (*S*,*S*)-AdoMet (with a half-life time of 12 h), and eventually, the entire supply will be consumed (although some loss to MTA will be unavoidable). To the extent that in vivo racemization of AdoMet competes with its utilization, (*R*,*S*)-AdoMet might be thought of as a slow-release, storage form of AdoMet. It is conceivable that if a sudden, large demand on the AdoMet supply were introduced, the re-formation of (*S*,*S*)-AdoMet from (*R*,*S*)-AdoMet might come to limit the transmethylation rate, at least locally. A binding protein might stabilize AdoMet against loss of chirality, as well as decomposition, although it is harder to imagine from a chemical point of view. To protect against MTA formation, it is sufficient to bind, say, the amino acid head and the sulfonium pole at mutually inaccessible positions. To protect against chirality loss, however, three sulfur ligands must be bound in place, including either the methyl group or the lone pair of electrons.

Thus, the instability of (*S*,*S*)-AdoMet with respect to hydrolysis and loss of chirality places before any organism that utilizes it strict demands in terms of immediate turnover or stabilization through macromolecular binding, if a serious waste of biosynthetic energy is to be avoided. The cellular economy has been forced to evolve within the limits of these demands.

Registry No. (*S*,*S*)-AdoMet, 78548-84-2.

References

Atkinson, D. E. (1977) *Cellular Energy Metabolism and Its Regulation*, p 75, Academic Press, New York.

Baddiley, J., Cantoni, G. L., & Jamieson, G. A. (1953) *J. Chem. Soc.*, 2662–2664.

Baldessarini, R. J. (1975) *Int. Rev. Neurobiol. 18*, 41–67.

Borchardt, R. T. (1979) *J. Am. Chem. Soc. 101*, 458–463.

Borchardt, R. T., & Wu, Y. S. (1976) *J. Med. Chem. 19*, 1099–1103.

Cantoni, G. L. (1952) *Johns Hopkins Univ., McCollum-Pratt Inst., Contrib. No. 23*, 129–152.

Cantoni, G. L. (1953) *J. Biol. Chem. 204*, 403–416.

Cantoni, G. L. (1960) in *Comparative Biochemistry* (Florkin, M., & Mason, H. S., Eds.) pp 181–241, Academic Press, New York.

Cornforth, J. W., Reichard, S. A., Talalay, P., Carrell, H. L., & Glusker, J. P. (1977) *J. Am. Chem. Soc. 99*, 7292–7300.

Coward, J. K., & Sweet, W. D. (1971) *J. Org. Chem. 36*, 2337–2346.

Coward, J. K., & Wu, F. Y.-H. (1973) *Anal. Biochem. 55*, 406–410.

Coward, J. K., Lok, R., & Takagi, O. (1976) *J. Am. Chem. Soc. 98*, 1057–1059.

Darwish, D. (1968) *Mech. React. Sulfur Compd. 3*, 33–35.

Darwish, D., & Tourigny, G. (1966) *J. Am. Chem. Soc. 88*, 4303–4304.

Darwish, D., & Scott, C. E. (1973) *Can. J. Chem. 51*, 3647–3658.

De la Haba, G., Jamieson, G. A., Mudd, S. H., & Richards, H. H. (1959) *J. Am. Chem. Soc. 81*, 3975–3980.

Duerre, J. A. (1981) in *Biochemistry of S-Adenosylmethionine and Related Compounds* (Usdin, E., Borchardt, R. T., & Creveling, C. R., Eds.) Macmillan Press, London.

Garbesi, A., Corsi, N., & Fava, A. (1970) *Helv. Chim. Acta 53*, 1499–1502.

Glazer, R. I., & Peale, A. L. (1978) *Anal. Biochem. 91*, 516–520.

Kunz, G. L., Hoffman, J. L., Chia, C.-S., & Stremel, B. (1980) *Arch. Biochem. Biophys. 202*, 565–572.

Mather, A. N. (1958) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 17*, 271.

Mudd, S. H. (1959) *J. Biol. Chem. 234*, 87–92.

Mudd, S. H., Ebert, M. H., & Scriver, C. R. (1980) *Metabolism 29*, 707–720.
Olsen, J., Wu, Y. S., Borchardt, R. T., & Schowen, R. L. (1979) in *Transmethylation* (Usdin, E., Borchardt, R. T., & Creveling, C. R., Eds.) pp 127–133, Elsevier/North-Holland, New York.
Parks, L. W., & Schlenk, F. (1958) *J. Biol. Chem. 230*, 295–305.
Savige, W. E., & Fontana, A. (1977) *Methods Enzymol. 47E*, 453–459.
Scartazzini, R., & Mislow, K. (1967) *Tetrahedron Lett. 28*, 2719–2722.
Schlenk, R., & Zydek-Cwick, C. R. (1969) *Arch. Biochem. Biophys. 134*, 414–422.
Sharpless, T. K., & Wolfenden, R. (1967) *Methods Enzymol. 12A*, 126–131.
Smith, J. D. (1976) *Biochem. Biophys. Res. Commun. 73*, 7–12.
Stolowitz, M. L., & Minch, J. J. (1981) *J. Am. Chem. Soc. 103*, 6015–6019.
Streitwieser, A. (1962) *Solvolytic Displacement Reactions*, p 13, McGraw-Hill, New York.
Swain, C. G., & Taylor, L. J. (1962) *J. Am. Chem. Soc. 84*, 2456–2457.
Usdin, E., Borchardt, R. T., & Creveling, C. R., Eds. (1979) *Transmethylation*, Elsevier/North-Holland, New York.
Usdin, E., Borchardt, R. T., & Creveling, C. R., Eds. (1981) *Biochemistry of S-Adenosylmethionine and Related Compounds*, Macmillan Press, London.
Yagi, K. (1971) *Methods Enzymol. 17B*, 608–622.
Zappia, V., Galletti, P., Olivia, A., & DeSantis, A. (1977) *Anal. Biochem. 79*, 535–543.
Zappia, V., Carteni-Farina, M., & Porcelli, M. (1979) in *Transmethylation* (Usdin, E., Borchardt, R. T., & Creveling, C. R., Eds.) pp 95–104, Elsevier/North-Holland, New York.

Escherichia coli Phosphoenolpyruvate-Dependent Phosphotransferase System: Stereospecificity of Proton Transfer in the Phosphorylation of Enzyme I from (*Z*)-Phosphoenolbutyrate†

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ABSTRACT: The stereochemistry of the proton transfer in the reaction of phosphoenolbutyrate with enzyme I has been established. During the reaction of the pure *Z* isomer of this analogue of phosphoenolpyruvate with enzyme I, to yield phosphoenzyme I and 2-oxobutyrate, the substrate is protonated at C-3 from the 2*re*,3*si* face. This stereospecificity was established for the transfer of a proton to (*Z*)-phospho[3-D]enolbutyrate and for the transfer of a deuteron to (*Z*)-phospho[3-H]enolbutyrate. The *E* isomer of phosphoenolbutyrate is not a substrate for enzyme I. Accordingly, the reaction of phosphoenzyme I with 2-oxobutyrate yields exclusively the *Z* isomer of phosphoenolbutyrate, and only the *pro-S* proton at C-3 of 2-oxobutyrate is abstracted. A kinetic H/D isotope effect of 6.8 in this reaction demonstrates the rate-limiting nature of the proton-transfer step. The stereochemical analysis of 2-oxo[3(*R*)-H,D]butyrate and of 2-oxo-[3(*S*)-H,D]butyrate was carried out by using the pyruvate kinase catalyzed enolization of this compound. This enzymatic enolization, with phosphate as a cofactor, is rapid at neutral pH and is a highly stereospecific reaction: only the *pro-R* proton at C-3 of 2-oxobutyrate is exchanged with solvent. This reaction was also used to generate the pure 3*R* and 3*S* enantiomers of 2-oxo[3-H,D]butyrate. The degree of protonation/deuteration at C-3 of 2-oxobutyrate was detected from the fine structure of the methyl proton nuclear magnetic resonance signal.

Enzyme I, a component of the bacterial PEP[1]-dependent phosphotransferase system, catalyzes the transfer of a phosphoryl group from PEP to a phosphocarrier protein HPr. This is the first step in a process that ultimately leads to the phosphorylation and concomitant transport of PTS sugars into the bacterial cell [for recent reviews, see Hays (1978) and Robillard (1982)]. The enzyme I catalyzed reaction proceeds via a phosphoenzyme intermediate (Stein et al., 1974; Waygood & Steeves, 1980; Saier et al., 1980; Hoving et al., 1981). After phosphorylation of enzyme I by PEP, ketopyruvate is the product species that dissociates from the phosphorylated enzyme. It is still unclear whether the enolate of pyruvate is an enzyme-bound intermediate during the reaction or if phosphoryl-group transfer and protonation take place in a concerted fashion. It has been clearly demonstrated, however, that the proton transfer does take place on the enzyme and that the base on enzyme I that is responsible for this proton transfer is not in protonic equilibrium with the solvent during the course of a reaction (Hoving et al., 1981). Since the protonation of the enol substrate is carried out by the enzyme, the reaction may be expected to take place stereospecifically. Thus, information on the extent to which the proton transfer

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[1] Abbreviations: PEP, phosphoenolpyruvate; PEB, phosphoenolbutyrate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; DTT, dithiothreitol; NMR, nuclear magnetic resonance; 2-OB, 2-oxobutyrate; D, ^{2}H; T, ^{3}H.

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