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15. Buildup rates obtained in ^2H_2O were calibrated against Tyr2,6-Tyr3,5 NOEs (2.45 Å). Buildup rates in ^1H_2O involving NH protons were calibrated against the sequential NH_i-NH_{i+1} distance in α-helical regions [2.8 Å (*9*)]. Ten percent of the calculated distance, representing the experimental uncertainty, was added to the upper bound.
16. The pseudo-atom corrections were 1.0 Å for methylene and methyl groups, 1.5 Å for Leu dimethyl groups, and 2 Å for Phe and Tyr 3,5 and 2,6 protons.
17. Upper and lower distance bounds for the amide H and carbonyl O atoms were 2.3 and 1.85 Å, respectively.
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Methyl Chloride Transferase: A Carbocation Route for Biosynthesis of Halometabolites

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Enzymatic synthesis of methyl halides through an *S*-adenosyl methionine transfer mechanism has been detected in cell extracts of *Phellinus promaceus* (a white rot fungus), *Endocladia muricata* (a marine red algae), and *Mesembryanthemum crystallium* (ice plant). This mechanism represents a novel pathway for the formation of halometabolites. The Michaelis constants for chloride and bromide ion and for *S*-adenosyl methionine in the reaction have been determined for the enzyme from *E. muricata*. A recent survey of marine algae indicates that there may be a broad distribution of this enzyme among marine algae.

THE MOST ABUNDANT HALOHYDROcarbon species in the upper atmosphere is methyl chloride, and it is widely believed that biological synthesis is largely responsible for sustaining a global emission rate estimated to be 5×10^6 tons of methyl chloride per year (*1*). The synthesis of methyl chloride by cultures of wood rot fungi has been well documented (*2*), and there have been isolated studies reporting the in vivo synthesis of methyl halides by marine macroalgae and phytoplankton (*3*). However, in vitro methyl chloride synthesis has not been reported. The established enzymatic mechanism for the biosynthesis of halometabolites involves the hydrogen peroxide–dependent oxidation of halides to form electrophilic halogenating species (*4*). The electrophilic halogen intermediate generated in the peroxidase-reaction can react with a broad spectrum of nucleophilic acceptors to form the halometabolites. In our laboratory we demonstrated the synthesis of one prevalent atmospheric halohydrocarbon, methyltribromide, through the peroxidatic route (*5*). In the synthesis of this compound, bromoperoxidase first catalyzes the multiple bromination of an activated methylene carbon atom adjacent to a ketone function (*6*). The enzymatic bromination reaction is followed by the nonenzymatic hydrolysis of the tribrominated methyl group to release methyltribromide in a classical bromoform reaction. However, all of our attempts to detect monohalomethanes through chloro- or bromoperoxidase-type reactions have been unsuccessful. Thus we have recently turned our attention to a potential alternative route for the synthesis of methyl halides. White (*7*) has shown that when the fungus *Phellinus pomaceus* is grown on deuterium-labeled glucose, serine, or methionine, the methyl chloride produced is also labeled. This observation is consistent with the methyl chloride being derived from methionine. A likely route for this reaction would be through the methyl donor, *S*-adenosyl methionine. We report the detection and partial purification of a methyl transferase that catalyzes the methylation of chloride, bromide, and iodide ions.

Methyl chloride transferase activity has been detected in cell-free extracts prepared from the fungus *P. pomaceus*, from the ma-

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Table 1. Methyl transferase activity levels in whole cells and crude cell extracts. The activity measurements for fungal cells were obtained by growing cultures in 100 ml of 5% malt extract, 100 mM KCl, 1% bactopeptone liquid media in sealed bottles. Gas samples were removed each day and analyzed as described in Fig. 1. Known amounts of methyl chloride in identically prepared bottles were used as standards. The value reported here was the amount of methyl chloride obtained during peak production periods. The values for whole cells of *E. muricata* and *M. crystallium* were obtained by incubating whole cells in 100 mM KCl in gas-tight vials. Gas samples were analyzed as in Fig. 1 with known amounts of methyl chloride as standards. The values for all crude cell extracts were obtained by incubating the cell extracts in 4-ml reaction mixtures containing 250 mM KCl, 500 μM SAM, 100 mM phosphate, pH 6.8. The *P. pomaceus* cell extracts were obtained by digesting cells with 10 mg of Novozyme 234 per milliliter for 1 hour followed by removal of cell debris by centrifugation. Cell extracts of *E. muricata* were obtained as in Fig. 1. Cell extracts of *M. crystallium* were obtained by grinding the cells in a Waring blender. Gas samples were analyzed in the same manner as those from whole cells.

Source	Methyl chloride production	
	Whole cells (pmol g^{-1} day^{-1})	Crude extract (fmol min^{-1} mg^{-1})
P. pomaceus	7	25
E. muricata	30	670
M. crystallium	19	3

rine algae, *Endocladia muricata*, and from the terrestrial succulent, *Mesembryanthemum crystallium*, commonly known as ice plant (*8*). Controls show that the presence of enzyme is necessary for methyl halide production. The synthesis of methyl chloride and other methyl halides was confirmed by gas chromatography–mass spectroscopy. All of the enzymes appear in the cytosolic fraction after cell disruption with a small amount of activity being pelleted with the cell debris. The rate of methyl chloride production found in the crude cell free extracts of each organism is compared with this same activity in whole cells in Table 1. The methyl chloride transferase has been purified 800-fold from extracts of *E. muricata*. The purification was achieved by an 80 to 100% ammonium sulfate precipitation step followed by a high-performance liquid chromatography (HPLC) gel filtration step on a 60 cm by 2.15 cm preparative Bio-Sil SEC-250 column. The column was eluted with 10 mM phosphate buffer, pH 7.0. The apparent molecular weight of the enzyme as determined by gel filtration is in the range of 20,000 to 25,000 daltons. No prosthetic group or cofactor requirement has been found for the *E. muricata* enzyme or the *P. pomaceus* enzyme. The partially purified enzyme catalyzes methyl bromide synthesis at a rate of 555 picomoles per minute per milligram of protein. This value compares favorably with other methyl transferase enzymes, which have specific activities in the range of 166 to 5800 picomoles per minute per milligram of protein (*9*).

The effect of pH and substrate concentrations on the algal enzyme is shown in Figs. 1 and 2. The enzyme shows maximal activity in the pH range 7.5 to 7.6. At pH values greater than 9.2, no methyl bromide production could be detected. The Michaelis constant K_m for bromide ion in the reaction is 40 mM, and the K_m for *S*-adenosyl methionine in the reaction is 16 μM. The K_m for chloride ion is approximately 5 mM.

As might be expected, the order of reactivity of the methyl transferase with different halide ions follows the order iodide, bromide, and chloride, with chloride being the poorest acceptor. This reactivity matches the expected order, since the rate of reaction of a carbocation with a halide should be dependent upon the nucleophilicity of the acceptor anion (*10*). Iodide is by far the preferred acceptor, the rate with iodide exceeding the bromide rate by more than two orders of magnitude. Fluoride is not a substrate. The rate of synthesis of the methyl halides at saturating concentrations of halides with identical enzyme concentrations is shown in Table 2. It has been proposed that atmospheric methyl chloride and methyl bromide are produced through the intermediate biosynthesis of methyl iodide (*11*). In this hypothesis, marine algae, phytoplankton, or both synthesize the methyl iodide, which then undergoes a chemical displacement reaction with chloride or bromide ions in seawater to produce the relevant methyl halide. Experiments with *E. muricata* fail to substantiate this theory, even though iodide is the best halide acceptor. When *E. muricata* whole cells or cell-free extracts are incubated in sea water reaction mixtures, methyl chloride is the only methyl halide that is formed. The enzymatic synthesis of methyl iodide and methyl bromide in seawater requires special supplementation of the reaction mixture with the appropriate halide anion.

The results reported in this paper, taken together with our recent survey of methyl chloride production by marine algae of the Monterey Bay area of California (*12*), suggests a broad distribution of the methyl chloride transferase enzyme. In the algae survey, 22 of 44 randomly collected algae

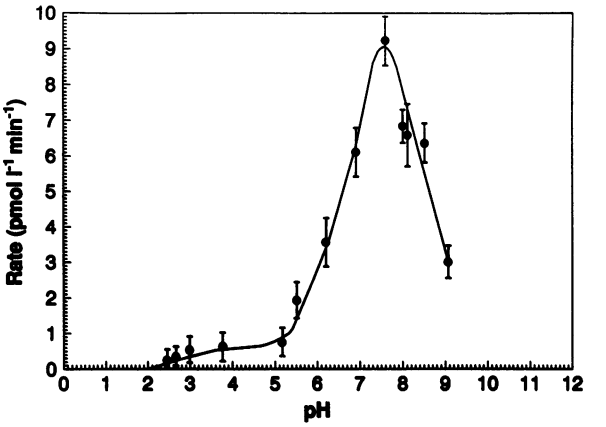

Fig. 1. The pH dependence of the rate of conversion of *S*-adenosyl methionine (SAM) to methyl bromide by the *E. muricata* enzyme. The pH dependence was examined by incubating crude cell extracts in the presence of bromide anion and SAM. Cell extracts were prepared by grinding whole cells in liquid nitrogen in a stainless steel Waring blender. The ground algae were suspended in 5 mM phosphate buffer, pH 6.7 (3 ml of buffer per gram wet weight algae), and the cell debris was removed by centrifugation. Aliquots of the cell extract (0.6 mg of protein) were added to 4-ml reaction mixtures containing 100 mM phosphate buffer at the desired pH, 250 mM KBr, and 500 μM SAM. Under these conditions after a short lag period, gas production was linear for at least 6 hours. The reaction mixtures were incubated at 25°C for 6 hours in gas-tight vials fitted with rubber stoppers. Gas samples were removed and analyzed by gas chromatography. Samples (1 ml) were assayed with a 6 foot by 1/4 inch (180 cm by 0.6 cm) glass column packed with 60/80 mesh carbopack, 1% SP 1000 in a Varian 3700 gas chromatograph interfaced with an SP 4290 computing integrator. At a column temperature of 60°C and a hydrogen flow rate of 30 ml min^{-1}, methyl bromide had a retention time of 0.83 min. Gas production was quantitated by relating the peak size from unknown samples to samples from identically prepared vials containing known amounts of methyl bromide. Three independent cell extracts were prepared and two aliquots from each preparation were averaged (a total of six replications); error bars are 1 SD.

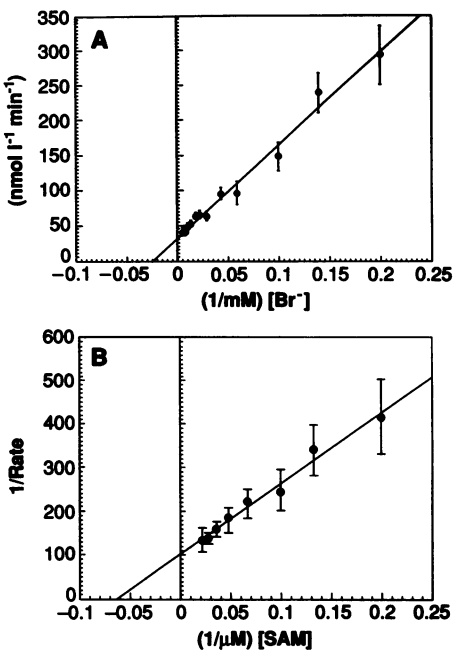

Fig. 2. The effect of substrate concentrations on methyl bromide production measured as the rate of SAM conversion. (**A**) The effect of bromide concentration. Whole cells were ground in liquid nitrogen. The ground algae were suspended 10 mM phosphate buffer, pH 7.58 (3 ml of buffer per gram wet weight of algae). Aliquots of the cell extract obtained by removal of the cell debris by centrifugation (0.6 mg of protein) were added to 4-ml reaction mixtures containing KBr that varied in concentration from 5 to 200 mM, 10 mM phosphate, pH 7.58, and 500 μM SAM. The reaction mixtures were incubated at 25°C for 6 hours. Gas samples were removed and analyzed as in Fig. 1. Results of two independently prepared cell extracts with two aliquots at each bromide concentration (a total of four replications) were averaged and plotted in a standard double reciprocal plot; error bars are ±1 SD. (**B**) The effect of SAM concentration. Cell extract was prepared as in (A). The extract (0.6 mg of protein) was added to 4-ml reaction mixtures containing SAM that varied in concentration from 1 to 45 μM, 10 mM phosphate, pH 7.58, and 250 mM KBr. The reaction mixtures were incubated at 25°C for 3 hours. Gas samples were removed and analyzed as in Fig. 1. Results of three independently prepared cell extracts with three aliquots at each SAM concentration (a total of nine replications) were averaged and plotted in a standard double reciprocal plot; error bars are ±1 SD.

Table 2. Comparison of the production of different methyl halides by methyl chloride transferase from *E. muricata*. Crude extract, prepared as in Fig. 2, was incubated in 4-ml reaction volumes containing 250 mM of the appropriate potassium halide, 10 mM phosphate, pH 7.58, and 500 μM SAM. Reactions were incubated at 25°C for 6 hours. Gas samples were removed and analyzed as in Fig. 1 except the appropriate methyl halide was used as a standard. The synthesis of all methyl halides was confirmed by gas chromatography–mass spectroscopy.

Methyl halide	Rate of methyl halide production (pmol liter^{-1} min^{-1})
CH_3Cl	8
CH_3Br	16
CH_3I	2165

were identified as methyl chloride producers. Thus it is likely that the methyl transferase enzyme is a constitutive activity in a variety of microorganisms and marine algae. The presence of the enzyme in ice plant, a terrestrial plant which grows in great abundance in the California coastal soils, is an interesting observation that perhaps signals a need for a survey of methyl chloride transferase activity in other succulents that grow in saline-rich environments. Also noteworthy is the fact that ice plant has a wide global distribution. Although the production of 5×10^6 tons per year represents a prodigious rate of methyl chloride synthesis, this number may be quite understandable in terms of the large terrestrial and marine biomass that can contribute to its formation.

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Identification of Small Clusters of Divergent Amino Acids That Mediate the Opposing Effects of *ras* and K*rev*-1

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K*rev*-1 is an anti-oncogene that was originally identified by its ability to induce morphologic reversion of *ras*-transformed cells that continue to express the *ras* gene. The K*rev*-1–encoded protein is structurally related to Ras proteins. The biological activities of a series of *ras*–K*rev*-1 chimeras were studied to test the hypothesis that K*rev*-1 may directly interfere with a *ras* function. The *ras*-specific and K*rev*-1–specific amino acids immediately surrounding residues 32 to 44, which are identical between the two proteins, determined whether the protein induced cellular transformation or suppressed *ras* transformation. Because this region in Ras proteins has been implicated in effector function, the results suggest that K*rev*-1 suppresses *ras*-induced transformation by interfering with interaction of Ras with its effector.

INTERPLAY BETWEEN POSITIVE AND negative regulators determines whether a cell will grow and divide, with oncogenes stimulating and anti-oncogenes (tumor suppressor genes) inhibiting these processes. Considerable insight has been gained into the mechanism by which oncogenes stimulate cell proliferation; however, much less is known about mechanisms of anti-oncogene function (*1*). Although the proteins of some anti-oncogenes are structurally unrelated to those of oncogenes, the K*rev*-1 anti-oncogene (*2*) encodes a protein that is structurally related to the *ras* oncogene–encoded proteins (*3*), with which it shares about 50% sequence similarity (*4, 5*). As is true of *ras*-encoded proteins, the K*rev*-1–encoded protein binds guanine nucleotides and possesses a guanosine triphosphatase (GTPase) activity (*4*).

K*rev*-1 induces morphological reversion of a cell line transformed by a *ras* oncogene. Because the reverted line still expresses the transforming *ras* gene, K*rev*-1 may inhibit *ras* function by interfering with some aspect of the *ras* pathway (*2*). To test this hypothesis, we identified the sequences in *ras* and K*rev*-1 that account for their opposing biological activities. If the critical differences between the two proteins were limited to a few amino acids, it would focus attention on the function served by this region. Therefore we have made a series of *ras*–K*rev*-1 chimeric genes and studied their ability to induce cellular transformation and to suppress *ras*-induced transformation.

The proteins encoded by K*rev*-1 and *ras* are 184 and 189 amino acids, respectively (Fig. 1). Sequence alignment indicates that the two proteins are colinear, with K*rev*-1 encoding two additional amino acids between *ras* residues 120 to 136, and seven fewer amino acids between *ras* residues 161 to 186. Compared with K*rev*-1, 70% of the NH_2-terminal 60 amino acids encoded by ras^H are identical, 60% of the next 60 residues are identical, and 33% of the COOH-terminal residues are identical.

The highly transforming v-*ras*-AT gene that we used for our chimeric constructions was derived from the Harvey murine sarcoma virus oncogene (*6, 7*) (Fig. 1). The protein product of v-*ras*-AT differed from c-*ras* by only two amino acids, a highly activating Arg^{12} in place of Gly, and Thr^{61} in place of Gln (Fig. 1). To facilitate construction of *ras*–K*rev*-1 chimeric genes, we engineered restriction endonuclease cleavage sites at comparable regions of each gene. This change resulted in restriction endonuclease sites at nucleotides encoding amino acids 5 (Hind III), 60 (Pst I), 109 (Aat II), and 146 (Hae II). In *ras*, the Hind III site is present in the wild-type gene, and the mutations that created the Aat II and Hae II sites were silent. The mutations required to make the Pst I site changed Thr^{59}, which is specific to v-*ras*, to Ala, the amino acid encoded by c-

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