Widespread occurrence of 2-acetylthiazole-4-carboxylic acid in biological material

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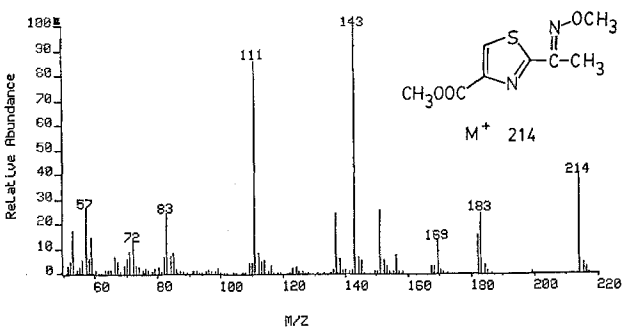
Summary. 2-Acetylthiazole-4-carboxylic acid was shown to be widely distributed in all organisms tested, which included members of the eukaryotes, archaebacteria, and eubacteria. This thiazole, which was identified and quantitated as the methyl ester methoxyamine derivative, was found in these organisms at levels of from 27 to 1100 nmol/g dry weight (d.wt) of tissue. On the basis of its widespread occurrence, the levels at which it occurs in these organisms, and its chemical structure, which contains a reactive carbonyl group, it is proposed that this compound is a previously undescribed coenzyme.

Key words. 2-Acetylthiazole-4-carboxylic acid; coenzymes; distribution.

During a recent survey, in which gas chromatography-mass spectrometry (GC-MS) was used to measure the distribution of alpha-ketodiacids in hot water extracts of various biological samples, an unknown peak was consistently observed in all of the assayed samples[1]. The highest mass ion (*m/z* 214) in the mass spectrum of this peak (fig.) was thought to be the molecular ion. Considering the method used to extract and derivatize the sample, which should have generated a methyl ester, the presence of a fragment ion at *m/z* 183, corresponding to M^+-31, indicated the presence of a methyl ester. The presence of an ^{34}S isotope ion in all of the major ions in the mass spectrum, i.e., ions 214, 183, 151, 143, and 111, indicated that the molecule contained one sulfur that was retained in all of these major fragments. Preparation of the derivative with 3 M HCl in [*methyl*-$^2H_3$]-methanol instead of diazomethane, or ethoxyamine instead of methoxyamine, increased the molecular ion mass by 3 and 14 *m/z*, respectively. These results confirmed that the parent molecule contains one carbonyl group, which reacts with alkoxyamines, and one carboxylic acid. Since the derivatized molecule, which incorporated a single nitrogen during the derivatization, had an even mass, the parent molecule must have had an odd number of nitrogens. GC-high resolution MS of the peak established a mass of 214.044, which corresponds to an elemental formula of $C_8H_{10}N_2O_3S$ (calculated mass 214.042). The base ion (*m/z* 143) in the mass spectrum must then correspond to $C_5H_5NO_2S$ and be generated by the loss of *m/z* 71 ($\cdot OCH_3 + C_2H_2N$) from the molecular ion. The loss of CH_3OH from the *m/z* 143 ion would account for the *m/z* 111 ion.



Mass spectrum of the methyl ester methoxyamine derivative of 2-acetylthiazole-4-carboxylic acid.

The observation that the underivatized molecule contains one nitrogen and one sulfur suggests that the molecule may contain a thiazole ring. If correct, then, on the basis of the constraints of the molecular formula, the complete molecule must contain a methylene or methyl carbon, a carbonyl group, a carboxylic acid, and a thiazole ring.

The pK_a of thiazole (2.53) is lowered by the conjugation of the ring with electron-withdrawing substituents such as carbonyl groups[2,3]. Thus, if the isolated compound extracted from 0.1 M HCl during the preparation of the derivative contains a thiazole, it must be substituted in such a way that the resulting thiazole would have a pKa of less than 1. Since the introduction of a carbonyl group at the 2 or 5 position of the thiazole ring lowers the pKa to 1–2, both carbonyl groups in the structure must be conjugated to the thiazole ring. Assuming that the structure does not contain an aldehyde, this would leave the six isomers of acetylthiazole-carboxylic acid and the six isomers of oxocarboxymethylmethylthiazole as possible structures.

Of the former group of structures, only 2-acetylthiazole-4-carboxylic acid makes sense biochemically in that one can readily see how this thiazole could arise by the condensation of cysteine with pyruvate. In the latter group, 4-methyl-α-oxo-5-thiazoleacetic acid could be derived from the 4-methyl-5-(β-hydroxyethyl)thiazole moiety of thiamine by the oxidation of the hydroxyethyl side chain. This compound has, in fact, been tentatively identified as a metabolite of the thiazole-containing drug clomethiazole[4], however, it was eliminated from consideration since the published mass spectrum of the methyl ester of this compound did not correspond to that of the methyl ester of the compound isolated from the cells.

Synthesis of 2-acetylthiazole-4-carboxylic acid, as described by Brooks and co-workers[5], derivatization, and

GC-MS analysis, as described for the unknown, showed that the products were identical. The only described occurrence of this compound is as a degradation product of the pheomycin-bleomycin group of antibiotics[6]. A closely related compound, 2-(alpha-aminoethyl)-thiazole-4-carboxylic acid, has also been identified as a component of the alkaloid N-3'-β-indolylethyl-2-alpha-aminoethylthiazole-4-carboxamide, which was isolated from *Thermoactinomyces* strain TM-64[7].

Analysis of the occurrence of 2-acetylthiazole-4-carboxylic acid in a wide range of organisms showed this thiazole to be present in all cells assayed (table). Quantitation of the compound, using GC-MS with selective-ion recording and [4,4,6,6-$^2H_4$] alpha-ketosuberic acid as an internal standard, indicated levels of from 27 to 1100 nmol/g d.wt of the thiazole in all of the cells assayed (table). The observed levels of 2-acetylthiazole-4-carboxylic acid are similar to those of many of the coenzymes present in many different types of cells[8,9].

Since the extraction and derivatization procedure used above had several rather harsh chemical steps that could possibly have led to the formation of 2-acetylthiazole-4-carboxylic acid by nonbiochemical means, it was necessary to prove that the 2-acetylthiazole-4-carboxylic acid was actually present in the cells before extraction and derivatization. This was accomplished by confirming the presence of 2-acetylthiazole-4-carboxylic acid in cells that were extracted with methanol at 3 °C. The 2-acetylthiazole-4-carboxylic acid was confirmed in the methanol extracts by GC-MS identification of its methyl ester, which was formed by reaction of the extract with diazomethane.

As discussed above, the 2-acetylthiazole-4-carboxylic acid could arise from the condensation of pyruvate with cysteine, with the C-2 of the thiazole arising from the C-1 of the pyruvate. Evidence in support of this idea comes from a biosynthetic experiment which resulted in the incorporation of two intact $^{13}C_2$-acetate units into the 2-acetylthiazole-4-carboxylic acid when cells of rumen methanogenic bacteria strain 10–16B were grown with [1,2-$^{13}C_2$]acetate. Since both the C-2 and C-3 of pyruvate and cysteine are biosynthetically derived as a unit from acetate in these cells[10], it is possible that the biosynthesis of the thiazole could proceed by the cyclization and subsequent dehydrogenation of either S- or N-pyruvoylcysteine.

The cyclization of cysteine peptides to form thiazolines and their subsequent dehydrogenation to form thiazoles is well documentated[11,12]. Many thiazole-containing natural products appear to be formed in this manner[13,14]. If a given peptide or protein contains an alanyl-cysteinyl sequence, then this process would lead to the generation of a peptide or a protein that contains a thiazole at the position of the original cysteine. Cleavage of the peptide would then release 2-(1-aminoethyl)thiazole-4-carboxylic acid which would lead to 2-acetylthiazole-4-carboxylic acid after transamination. However, if this mechanism were responsible for the nonenzymatic generation of the thiazole, then it would occur at all of the cysteine residues in proteins and produce a wide range of different substituted thiazoles, e.g., 2-formylthiazole-4-carboxylic acid from glycyl-cysteine and 2-isobutyrylthiazole-4-carboxylic acid from valinyl-cysteine. A search of the GC-MS data from the above samples, however, showed none of these homologues to be present, which would indicate that the thiazole is not formed by the random decomposition of cysteine-containing peptides or proteins.

From the above analyses, one can surmise that this thiazole is present in all organisms to carry out a specific function(s). In the bacteria assayed, this thiazole is clearly generated by the cells since it was not present in the growth media. For rat liver, the origin of the thiazole is more complex; the compound could either have been generated in the liver, produced by the bacteria present in the rat, and/or have been present in the food consumed by the rat.

One possible explanation for the wide occurrence of this thiazole, as supported by its structure, is that it is a previously unrecognized coenzyme. The molecule is cationic, as are all coenzymes, and it contains an active carbonyl group, which could function like other carbonyl-containing coenzymes in nitrogen metabolism,

Occurrence of 2-acetylthiazole-4-carboxylic acid in biological samples

Organism/tissue	nmol/g d.wt
Rat liver	210
E. coli B grown on glucose	320
E. coli B grown on amino acids	992
Saccharomyces cerevisiae	27
Bacteroides fragilis	1100
Hemophilus influenzae	540
Deinococcus radiodurans	33
Sulfolobus solfataricus	150
Methanobacterium thermoautotrophicum ΔH	655
Methanococcus vannelli	720
Rumen strain 10–16B	180
Halobacterium halobium	~100

The bacterial cells were grown under a wide range of conditions as previously described[1]. The rat liver was obtained from a freshly killed rat and *Escherichia coli* B was grown on a minimal salts medium with glucose or casamino acids as the carbon source[16]. Tissue samples (1–2 g wet weight) were suspended in 5 ml of 0.04 M phosphate buffer and a known amount of [4,4,6,6-$^2H_4$] alpha-ketosuberic acid was added as an internal standard. The resulting suspension was heated for 20 min at 100 °C, cooled, and centrifuged ($39 \times 10^3 \times g$, 10 min) to remove insoluble material. The resulting clear liquid was removed, mixed with 20 mg of methoxyamine hydrochloride, and the resulting solution adjusted to pH 13 by the addition of 1 N NaOH. The solution was then concentrated to 2 ml by evaporation with a stream of nitrogen while maintained at > 90 °C. The cooled solution was saturated with $(NH_4)_2SO_4$ and extracted (3×) with 2-ml portions of methyl acetate which were discarded; the resulting aqueous layer was adjusted to pH 1–2 by the addition of 6 M HCl and the solution extracted (3×) with 1-ml portions of methyl acetate. The combined extracts were evaporated to dryness, dissolved in methanol, converted to their methyl esters by reaction with an excess of diazomethane in diethyl ether, and the resulting O-methyoxamine derivatives were purified by preparative TLC on silica gel using methylene chloride-methyl acetate (9:1) as solvent. In this solvent system, the derivatives of both the alpha-ketosuberic acid and the 2-acetylthiazole-4-carboxylic acid had an R_f of 0.53. Analyses (GC-MS) of the purified derivatives were performed as previously described[17].

e.g., pyridoxal-P and the pyruvate in the pyruvoyl coenzymes [15].

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Inhibitory effects of spermine and spermidine on muscle calpain II

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Summary. The muscle enzyme calpain II, in contrast to muscle calpain I, was markedly inhibited by millimolar concentrations of the polyamines spermine and spermidine. These compounds and the calpain inhibitor calpastatin had synergistic inhibitory effects on calpain II. These results suggest that the polyamines may have possible regulatory effects on the in vivo activity of calpain II enzymes.
Key words. Calpain; calcium-activated neutral proteinase; spermine; spermidine.

Polyamines such as spermine and spermidine are the most abundant intracellular organic cations in eucaryotes, and their concentrations, which can be changed by a number of factors [1, 2], may be as high as 10 mM in different cell compartments and at certain phases of the cell cycle [3]. Interest has recently centered on their possible roles as modulators of cellular processes through their effects on intracellular calcium levels [4] and the activities of calcium-dependent enzyme systems [5] and calmodulin [6]. An interesting group of calcium-dependent enzymes is the calpains [7] (calcium-activated neutral proteinases) which are intracellular thiol proteinases containing calmodulin-like sequences [8]. The calpains have been implicated in a number of important intracellular processes, and like the calmodulins, their activities can be inhibited by a number of pharmacological agents [9–11]. Because of the effects of spermine and spermidine on calmodulin, and the fact that calpains are sensitive to effectors of calmodulin, we have investigated the effects of spermine and spermidine on different calpains in order to determine if in vivo calpain activity may be affected by the polyamines.

Materials and methods

Calpain I was prepared from chicken gizzard smooth muscle using Reactive Blue 2-Sepharose Cl-6B chromatography [12], after which active fractions from this column were fractionated on a Pharmacia FPLC MonoQ column in 1 mM dithiothreitol, 1 mM [ethylenebis (oxyethylenenitrilo)] tetraacetic acid, 0.01% NaN_3, 50 mM Tris, pH 8.0. Pure enzyme was eluted at approximately 0.35 M when the column was developed with a 0–0.5 M NaCl gradient. Calpain II was purified from chicken gizzard [13] and from mixed skeletal hamster muscle [14]. Calpastatin was obtained from chicken gizzard smooth muscle, and after heat treatment [9], active fractions were purified to homogeneity on a 5 × 60-cm Superose 12 column of a Pharmacia FPLC system. Assays of calpains were performed at the minimal Ca^{2+} concentrations necessary for maximum activity (5 μM for calpain I and 2.5 mM for calpain II) with purified desmin as the substrate for calpain I [15] and azocasein as the calpain II substrate [16]. In experiments on calpain in the presence of calpastatin, an amount of calpastatin was used in the assays which gave approximately half maximal inhibi-