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Biosynthesis of Ergothioneine from Endogenous Hercynine in *Mycobacterium smegmatis*

DOROTHY S. GENGHOF AND OLGA VAN DAMME

Department of Microbiology and Immunology, Albert Einstein College of Medicine, Yeshiva University, New York, New York 10461

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Ergothioneine was synthesized and accumulated in growing cultures of *Mycobacterium smegmatis* when the medium was adequately supplied with sulfur. In a low sulfur medium, the accumulation was sharply limited although growth of the organism was apparently normal. Synthesis of hercynine, the precursor of ergothioneine, was unaffected by low sulfur levels and was markedly increased by addition of L-histidine, the precursor of hercynine. Resting-cell pellicle experiments, performed with cells grown on the low sulfur high histidine medium, showed that ergothioniene was synthesized from endogenous hercynine, when cysteine or compounds readily converted to cysteine (such as cystine, lanthionine, cystathionine, and thiazolidine carboxylic acid) were added. Homocysteine and djenkolic acid allowed for minimal synthesis of betaine, whereas methionine, *S*-methylcysteine, sodium sulfate, and sodium thiosulfate were unable to donate sulfur for ergothioniene synthesis under the experimental conditions employed. Addition of cysteine to a resting pellicle preparation caused the formation of 100 to 200 μg of ergothioneine per g of dry cells in 2.5 to 3 hr. A modified procedure for isolating ergothioneine and hercynine, employing a 75% ethyl alcohol extraction of wet organisms, followed by a single alumina column separation of the compounds, is described.

De novo synthesis of ergothioneine and hercynine from radioactive precursors was shown to occur in growing cultures of *Mycobacterium smegmatis* (4). It was established in *Neurospora crassa* (1) that the final step in synthesis involves the addition of sulfur to hercynine. The importance of cysteine in promoting this synthesis was demonstrated by Melville et al. (5) in competition experiments involving suppression of ^{35}S-sulfate incorporation into ergothioneine. In the present study, the capacity of a variety of sulfur donors to permit synthesis of ergothioneine from endogenous hercynine, in a resting pellicle preparation of *M. smegmatis*, was investigated, and the quantitative relationships of the betaines to sulfur levels in the medium were explored. In addition, the optimal time and temperature for synthesis in the resting cells was established.

MATERIALS AND METHODS

A stock strain of *M. smegmatis* (Cornell University Medical College) was carried on slants of American Trudeau Society Medium (8) and was grown as a pellicle on Sauton medium (7). During most of the study, Sauton medium, adjusted to *p*H 7.2, was modified to lower the sulfur content: $MgSO_4 \cdot 7H_2O$ was replaced by an equivalent amount of $MgCl_2$, and sulfur was added as $(NH_4)_2SO_4$, 0.76 mM. This permitted good growth of the organisms but reduced the ergothioneine content to a low level after several days.

In experiments with growing cultures, the mycobacteria were propagated on the surface of 100 ml of medium in Roux flasks at 37 C. In experiments involving unmodified Sauton medium, the organisms were washed, dried in a vacuum oven at 80 C for 3 to 4 hr, weighed, and extracted with hot water (4). However, in all other cases, extractions were made by treating the washed, triturated wet organisms with 75% ethyl alcohol (three extractions of 1.5 hr each at room temperature). The alcoholic extract was dried, and the residue was taken up in 80% ethyl alcohol and fractionated on an alumina column. The ergothioneine and hercynine components were effectively separated by this method, with considerable saving of time and effort as compared to an earlier technique (4). After column separation, the individual 5-ml effluent fractions were divided into 2.5-ml portions; one portion was diazotized and tested for ergothioneine, as previously described (4). Hercynine assay, by use of the modified method described below, was performed upon the remaining 2.5-ml effluents from tubes which showed the color of diazotized hercynine in the ergothioneine test (fractions 6-10). Although effluents of fractions 4 and 5 sometimes showed color suggestive of hercynine upon diazotization, the com-

ponent present in these tubes did not migrate as hercynine when examined by paper chromatography. For final calculations, the dry weight of the alcohol-extracted cells was used.

For resting-cell experiments, organisms were grown as pellicles on 100 ml of modified Sauton medium (containing 0.76 mM sulfur and 10 mM L-histidine) at 37 C for 12 or 13 days. The culture fluid was poured off, the under side of the pellicle was washed gently with saline, and the saline was replaced with 99 ml of 0.1 M phosphate buffer (*p*H 6.6) and 1 ml of solution made from the sulfur compound to be tested. Test flasks were incubated at 37 C for 2.5 or 3 hr and were then placed in an ice bath to arrest metabolic processes. Cells and fluid were separated as soon as possible, the recovered fluids were frozen, and the alcoholic cell extracts were stored at 4 C, until processing. Extracts and fluids were dried under vacuum (70 C), prior to analysis for ergothioneine and hercynine.

Hercynine assay. A standard of pure L-hercynine (20 μg), prepared by D. B. Melville, was treated, along with the samples to be measured, in an ice bath with cold diazotized sulfanilic acid (the color was allowed to develop for 10 min, in contrast to 45 sec for ergothioneine) before the addition of strong NaOH to stop the reaction (4). After 10 min at room temperature, the intensity of the yellow to orange colors of the diazotized hercynine was measured in a photometer (525 mμ). This modified assay method gave yields amounting to 90 to 100% recovery of the compound, following fractionation on an alumina column by use of either 75 or 80% ethyl alcohol as the solvent.

Ergothioneine and hercynine recoveries. Addition of pure ergothioneine and hercynine to cell extracts and buffer fluids yielded recoveries of 70 to 85% and 87 to 105%, respectively. Pure ergothioneine, chromatographed on alumina by use of 80% ethyl alcohol, consistently yielded only an 80% recovery, as compared to a yield of 95 to 100% with 75% ethyl alcohol as the eluting agent. However, despite the reduced yield, 80% ethyl alcohol was used to allow separation of the betaines. Recoveries for duplicate determinations varied by ±3 to 5% for ergothioneine and ±10 to 15% for hercynine.

RESULTS

The relationship between ergothioneine and hercynine accumulations in *M. smegmatis* and the level of sulfur in the growth medium is shown in Fig. 1. When complete Sauton medium, containing 2.06 mM sulfur, was used, substantial amounts of ergothioneine and moderate amounts of hercynine accumulated during a 2- to 3-week growth period. However, in the medium modified by reduction of sulfur to 0.76 mM, the small amounts of ergothioneine that were formed in the beginning essentially disappeared after the fifth day; levels of hercynine were only slightly lower than in the complete medium. The reduced sulfur level did not appear to impair the character of

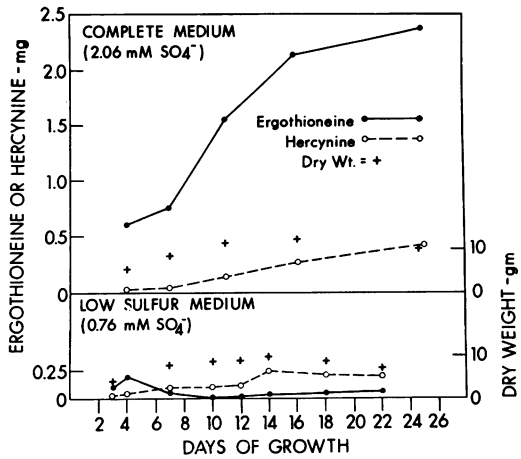

FIG. 1. *Accumulation of ergothioneine and hercynine in Mycobacterium smegmatis pellicles on Sauton medium containing normal and reduced sulfur levels.*

the growth, although yields of the bacteria were somewhat lower than for the complete medium. Further reduction of sulfur to 0.38 mM resulted in half the amount of growth, no detectable ergothioneine, and a marked reduction in hercynine at the end of a 12-day period.

When 10 mM L-histidine was added to the low sulfur medium, observations at 5, 10, and 21 days showed a quadrupling of hercynine, as compared to control cultures without histidine supplementation, and there was little if any increase in ergothioneine. Presumably, the unusual accumulation of hercynine was caused by lack of sulfur necessary for conversion to ergothioneine.

Resting cells, in the form of undisturbed pellicles, were found to show rapid synthesis of ergothioneine in the presence of a sulfur donor. A system of this type was examined to obtain information about conditions that would influence the synthesis. Cells were grown in the low sulfur medium, containing 10 mM L-histidine, to provide a low ergothioneine level and a high level of endogenous hercynine. A 12-day pellicle was used as the youngest capable of withstanding the physical manipulations necessary for the experiment.

The influence of time and temperature upon ergothioneine synthesis from endogenous hercynine, following the addition of L-cysteine to resting-cell preparations, is shown in Fig. 2. A small amount of synthesis was observed at 0.5 hr, and, by 2.5 hr, the content of ergothioneine in the system containing cysteine was six times that of the control without cysteine. When this 2.5-hr incubation time was used, maximal synthesis

was found to occur at 37 C, a modest amount at 23 C, and none at 45 C.

Synthesis of ergothioneine at 37 C (Fig. 2) has been observed over the range of *p*H 6.6 to 7.6. However, *p*H 6.6 was chosen for all subsequent experiments to help maintain cysteine in the reduced state.

The ergothioneine-synthesizing capacity of the pellicle, as compared to cell suspensions of *M. smegmatis*, was examined (Table 1). The pellicle proved to be greatly superior to the nonaerated suspension used in the first experiment. However, when the cell suspension was aerated, there was a definite increase in the amount of ergothioneine synthesized by the dispersed cells. Presumably, aeration promoted metabolic activity in this aerobic organism. This conclusion was supported by evidence from a supplementary resting pellicle experiment in which the use of Cleland's reagent (2), a strong reducing substance, lowered the level of ergothioneine synthesis by a considerable amount when compared to a control culture (8.4 versus 14.2 mg/100 g).

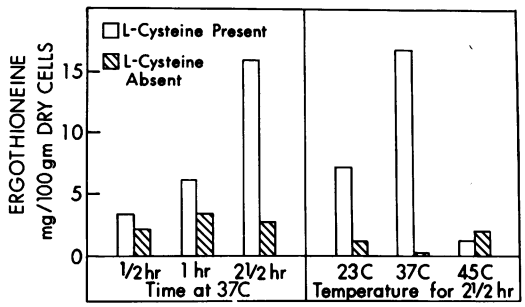

FIG. 2. *Influence of time and temperature on synthesis of ergothioneine in resting pellicle preparations. Twelve-day-old pellicles were prepared by decanting the culture fluid, washing with saline so as not to disturb the pellicle, replacing the wash solution with pH 6.6 buffer, and finally adding* L-*cysteine to a concentration of 5* mM.

The high levels of hercynine reflected the accumulation of the endogenous compound when *M. smegmatis* was grown in the presence of added histidine and a limited amount of sulfur. The variations in the total hercynine figures may reflect overall inaccuracies of the assay of fluids and cell extracts (*see* Materials and Methods).

The distribution of the betaines between the cells and buffer fluids shows that these substances may be present in both places, although sometimes they are found almost entirely within the cell. Whether or not synthesis actually takes place in the buffer has not yet been examined.

Earlier work by Melville et al. (5) indicated the effectiveness of cysteine, as compared to other sulfur compounds, in suppressing the incorporation of ^{35}S-sulfate into the ergothioneine of a growing culture of *N. crassa*. The resting pellicle system described here permitted direct measurement of amounts of ergothioneine synthesized from endogenous hercynine in *M. smegmatis*, when various potential sulfur donor compounds were added to the system. Eleven sulfur com-

TABLE 1. *Synthesis of ergothioneine by resting cell preparations of Mycobacterium smegmatis*

Expt no.	Cell state[a]	Compound added[b]	Distribution of the betaines (mg/100 g of cells)					
			Cells		Buffer fluid		Cells and buffer fluid	
			Ergothioneine	Hercynine	Ergothioneine	Hercynine	Ergothioneine	Hercynine
1	Pellicle	L-Cysteine	8	28	13.8	53	21.8	81
		None	0	45	0	23	0	68
	Suspension nonaerated[c]	L-Cysteine	0.5	24	2.8	59	3.3	83
		None	0	28	0	53	0	81
2	Pellicle	L-Cysteine	5.4	14	6.5	34	11.9	48
		None	0	23	0	35	0	58
	Suspension aerated[c]	L-Cysteine	7.2	44	1.0	20	8.2	64
		None	1.2	21	0	22	1.2	43

[a] Preliminary pellicular growth on medium containing 10 mM L-histidine, 0.76 mM $(NH_4)_2SO_4$; then the cells were incubated as pellicles or suspensions for 2.5 hr at 37 C in the presence of 100 ml of 0.1 M phosphate buffer.

[b] L-Cysteine added to a concentration of 5 mM.

[c] Cells from the pellicular growth of one flask were washed and suspended in the buffer. The nonaerated suspension was not stirred; a gentle air stream constantly bubbled through the aerated suspension.

pounds ranging from inorganic, such as sodium sulfate and thiosulfate, to organic, such as cysteine and cystathionine, were tested for this ability (Table 2).

The sulfur compounds tested may be grouped in order of ability to promote synthesis of ergothioneine. Cysteine, cystine, and lanthionine were the most active; cystathionine and thiazolidine carboxylic acid were moderately active; homocysteine and djenkolic acid were low in activity; and *S*-methylcysteine, methionine, sodium sulfate, and thiosulfate were completely inactive.

TABLE 2. *Capacity of various sulfur compounds to promote synthesis of ergothioneine from endogenous hercynine in resting cell pellicles of Mycobacterium smegmatis*[a]

Expt[b]	Compound added[c]	Content of cells + buffer fluid (mg/100 g of cells)		
		Ergothioneine	Hercynine	Total[d] ergothioneine + hercynine
1	L-Cysteine	28.6	39	68
	L-Cystine	20.0	49	69
	DL-Homocysteine	6.9	63	70
	L-Methionine	2.0	47	49
	$Na_2S_2O_3 \cdot 5H_2O$	3.7	65	67
	Na_2SO_4	1.5	62	66
	None	4.0	56	60
2	L-Cysteine	19.2	53	72
	L-Cystine	22.0	44	66
	L-Lanthionine	22.5	39	62
	L-Thiazolidine-4-carboxylic acid	17.2	50	67
	DL-Cystathionine	16.4	41	57
	L-Djenkolic acid	10.5	63	73
	S-methyl-L-cysteine	5.2	49	54
	None	7.8	58	64

[a] Pellicles grown for 13 days on medium containing 10 mM L-histidine, 0.76 mM $(NH_4)_2SO_4$; then incubated for 3 hr at 37 C on 0.1 M phosphate buffer.

[b] Experiment 1: ergothioneine and hercynine detected in both cells and buffer fluid. Experiment 2: essentially all ergothioneine and most of the hercynine detected in cells only. Dry weight range: experiment 1, 0.9 to 1.0 g per flask; experiment 2, 0.7 to 0.9 g. Most determinations were made in duplicate.

[c] Added as 5 mM equivalents of sulfur; DL compounds as 10 mM.

[d] Ergothioneine values represent 70 to 75% actual amount present (*see* Materials and Methods); hercynine values about 90% of actual amount, although the reproducibility of this value is sometimes variable.

Lanthionine and thiazolidine carboxylic acid were shown to exert a sparing effect on the need for methionine in *Tetrahymena geleii* (3). This effect was related to the ability of these compounds to be converted to cysteine by the organism, and, presumably, their sulfur-donating capacity in the present experiment was also related to this ability.

Cystathionine and homocysteine, intermediates after cysteine on the metabolic pathway to methionine in some microorganisms, were active in proportion to their proximity to cysteine along the pathway. Apparently methionine was not converted rapidly enough under our experimental conditions to be effective as a sulfur donor (assuming that rate of absorption of the methionine by the resting cells is not a factor).

S-methylcysteine was as inactive as methionine, although it seemed to be a likely potential sulfur donor for the reaction. This compound is a metabolite found in several strains of *N. crassa*, but it did not seem to function in that organism as a sulfur source through transformation to cysteine (6). Since cysteine or compounds rapidly converted to cysteine seemed to be the best sulfur donors for ergothioneine synthesis, the inactivity of *S*-methylcysteine as a sulfur source in our experiment is consistent with its performance in *N. crassa*.

In the resting-pellicle study, ergothioneine was synthesized from endogenous hercynine by the addition of sulfur-containing compounds. This finding is supported by comparing the relative levels of ergothioneine and hercynine found in cells plus fluid following synthesis. The lowest levels of hercynine were found where the greatest synthesis of ergothioneine occurred, whereas the highest levels of endogenous hercynine accompanied low levels of ergothioneine. Indeed, the sums of ergothioneine and hercynine yields, following incubation, were roughly equivalent for most of the compounds tested.

DISCUSSION

The present investigation shows that ergothioneine is produced and gradually accumulates over a long growth period in the presence of adequate amounts of sulfur (2.06 mM). Near maximal accumulation (2 mg/g of cells) was reached at 16 days, but, under conditions of reduced levels of sulfur (0.76 mM), the small amount of ergothioneine present in the organism at 3 to 4 days (0.2 mg/g of cells) fell off thereafter and remained at a low level. Despite the low sulfur level and concomitant low ergothioneine level,

the appearance of the organisms seemed unaffected, although there was a slight reduction in weight. Therefore, one may theorize that accumulations of ergothioneine are not essential for growth and the compound may act as a depot for sulfur. Accumulation of hercynine appeared unaffected by the amount of sulfur present when unmodified medium was used, but this accumulation can be increased as much as sixfold at 12 days (from 0.1 to 0.6 mg/g of cells) by reinforcing the low sulfur medium with 10 mM L-histidine. This finding emphasizes the role of histidine as a precursor for hercynine formation (1, 5) and shows that when sulfur is absent hercynine accumulates.

Data from the resting-cell experiments confirm the earlier observation of Melville et al. (5) that cysteine is an excellent sulfur donor for synthesis of ergothioneine from hercynine. Their studies involved inhibition of incorporation of ^{35}S-sulfate into ergothioneine in growing cultures of *N. crassa*, whereas in our experiments actual measurement of amounts of ergothioneine show unquestionably that synthesis of the betaine has occurred. Furthermore, other sulfur donors that are readily converted to cysteine by microorganisms such as cystine, cystathionine, lanthionine, and thiazolidine carboxylic acid seem to be about as effective in promoting synthesis. Those compounds not rapidly converted to cysteine, such as methionine, homocysteine, and *S*-methyl-cysteine, permit little if any ergothioneine synthesis.

Melville et al. (5) proposed the formation of an intermediary thio ether between cysteine and hercynine. Ban (Ph.D. Thesis, University of Southern California, Los Angeles, 1958) provided evidence for such a compound and named it *S*-alanylergothioneine. The development of a cell-free system of synthesis in *M. smegmatis* would permit testing of this compound, should it become available, and could lead to the elucidation of the chemistry of the reaction in this organism.

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