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Conversion of Histidine to Hercynine by *Neurospora crassa*

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Cell-free extracts of *Neurospora crassa* mycelium catalyze the methylation of L-histidine to form hercynine (histidine betaine). The partially methylated compounds, α-*N*-methyl-L-histidine and α-*N*,*N*-dimethyl-L-histidine, are also methylated by the same enzyme preparation to form hercynine. All three methylation reactions are stimulated by the addition of *S*-adenosylmethionine.

Hercynine was originally identified as a constituent of mushrooms (6), and its structure was established to be α-*N*,*N*,*N*-trimethylhistidine by synthesis from α-chloroimidazolepropionic acid and trimethylamine (2). Studies by Askari and Melville (1) on the biosynthesis of ergothioneine (2-mercaptohistidine betaine) in *Neurospora crassa* demonstrated the occurrence of hercynine in this fungus and its participation as an intermediate in the synthesis of ergothioneine. Ergothioneine is synthesized by a variety of fungi, but bacteria in general do not appear to synthesize the compound (9). However, Genghof and Van Damme (3, 4) showed that hercynine and ergothioneine are synthesized by mycobacteria. The work reported here was undertaken to establish the general nature of the enzymatic methylation reactions involved in the conversion of histidine to hercynine by *N. crassa*. A cell-free extract of *N. crassa* mycelium has been obtained which catalyzes the conversion of L-histidine to hercynine. The partially methylated compounds, α-*N*-methyl-L-histidine and α-*N*,*N*-dimethyl-L-histidine, are also converted to hercynine in the presence of the extract. When a dialyzed extract was used, hercynine synthesis from each of the three substrates was completely dependent upon the addition of *S*-adenosylmethionine.

MATERIALS AND METHODS

Hercynine was synthesized from L-histidine (11). Ergothioneine was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, and was recrystallized from aqueous ethyl alcohol. (−)*S*-adenosyl-L-methionine was used as the iodide (90% purity, Calbiochem, Los Angeles, Calif.). L-Histidine-UL-^{14}C was purchased from New England Nuclear Corp., Boston, Mass. Labeled α-*N*-methylhistidine, α-*N*,*N*-dimethylhistidine, and hercynine were prepared from ^{14}C-L-histidine by small-scale adaptations of the methods described for the synthesis of the nonisotopic compounds (11). ^{14}C-ergothioneine was prepared biosynthetically (1). Specific radioactivities were determined from thin layers prepared in planchets by the evaporation of solution samples containing approximately 10 μg of solids and were measured in an Omni-Guard (Tracerlab, Waltham, Mass.) thin-window gas-flow counter. Chemical and radiochemical purities of the compounds were established by paper chromatography.

Chromatography. Chromatographic analyses were carried out by the ascending method with Whatman no. 1 paper and *n*-tributylamine-ethyl alcohol-water (1:8:1, v/v/v), sulfuric acid-ethyl alcohol-water (1:80:20, v/v/v), or 0.1 M aqueous sodium acetate-ethyl alcohol (1:3, v/v) as the developing solvents, for 18 to 36 hr. Radioactive areas on the chromatograms were located with a Vanguard model 880 scanner, and the areas under the peaks were integrated by planimetry. The chromatograms were sprayed with diazotized sulfanilic acid and sodium carbonate (11) to establish the location of the imidazole derivatives; all of the histidine compounds used in this study, including hercynine and ergothioneine, give a positive test. Typical results obtained with mixtures of the pure labeled compounds are shown in Fig. 1. Separation of all five compounds could frequently be obtained after extended development with the tributylamine solvent. However, when a large amount of histidine, hercynine, or ergothioneine was present, these three compounds overlapped on the chromatograms. The most effective analysis of mixtures was achieved by the use of all three solvents. The tributylamine solvent was particularly useful for estimating methylhistidine and dimethylhistidine, the sulfuric acid solvent for ergothioneine, and the acetate solvent for histidine. Hercynine was estimated by difference; for example, by subtracting separately determined values for histidine and ergothioneine from the total value of a peak area containing histidine, hercynine, and ergothioneine.

Preparation of mycelial extract. *N. crassa* (ATCC 10336, wild type A) was grown from spore inocula in 100-ml portions of chemically defined medium (5) in 250-ml wide-mouth Erlenmeyer flasks on a rotary

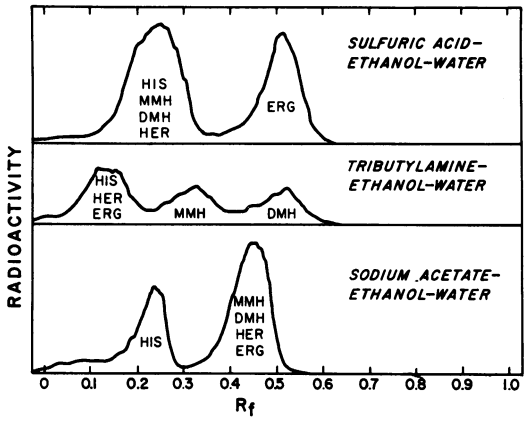

FIG. 1. *Separation of a mixture of ^{14}C-labeled compounds by paper chromatography in three solvents. Abbreviations: HIS, histidine; MMH, methylhistidine; DMH, dimethylhistidine; HER, hercynine; ERG, ergothioneine. Total radioactivity applied to each chromatogram was within the range of 7,000 to 10,000 counts per min.*

shaker at 30 C for 24 hr. Each 100-ml culture yielded 2 to 3 g of moist mycelium. A mycelial pad from 16 flasks was collected by filtration and was homogenized in a VirTis "45" homogenizer with 160 ml of cold 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, *p*H 8.5, for 10 min with the flask cooled in an ice bath. The homogenate was centrifuged at 30,000 × *g* for 30 min, and the supernatant solution was dialyzed against three 1-liter portions of 0.01 M Tris-hydrochloride buffer, *p*H 8.5, over a period of 24 hr. All operations were carried out at 2 to 5 C. The resulting solution contained 4 to 5 mg of protein per ml, as determined by the biuret test (7) with crystalline bovine plasma albumin as a standard, and was used for the enzymatic methylation studies. The preparation was stable for several weeks if kept at 5 C.

Enzymatic methylations. It was found that methylation of each of the labeled substrates was significantly stimulated by the addition of *S*-adenosylmethionine; this was particularly evident when dialyzed extracts were used as the source of enzyme. Excessive amounts of *S*-adenosylmethionine inhibited the methylations. The concentration chosen for routine use was that which provided almost maximal stimulation. The complete reaction mixture contained 0.6 μmole of *S*-adenosylmethionine, 0.2 μmole of substrate, and 4 ml of enzyme preparation, in a total volume of 5 ml. Enzymatic incubations were carried out at 25 C for 3, 6, or 12 hr, with essentially identical results. At the end of the incubation period, 20 ml of ethyl alcohol was added, the mixture was cooled, and insoluble material was separated by centrifugation. The supernatant solution was concentrated to dryness under reduced pressure, the residue was dissolved in 0.1 or 0.2 ml of 75% ethyl alcohol, and 25 or 50 μliters was used for paper chromatography.

RESULTS

The reliability of the analytical method was established by replicate control analyses carried out by chromatography of known mixtures of the pure ^{14}C-labeled compounds. Although ^{14}C-ergothioneine was not formed from hercynine during the enzymatic incubations, it was included in the control analyses to evaluate application of the method to systems such as whole mycelium in which ergothioneine is synthesized. Duplicate chromatographic analyses of a mixture of histidine, methylhistidine, dimethylhistidine, hercynine, and ergothioneine were run in the three different solvents, each at total radioactivity levels of 2,000, 4,000, and 8,000 counts per min. Recoveries for each compound were within the following ranges: histidine, 95 to 105%; methylhistidine, 94 to 104%; dimethylhistidine, 95 to 109%; ergothioneine, 91 to 105%; and hercynine (by difference), 82 to 123%. These recoveries are considered to be satisfactory for the present studies.

Table 1 shows the distribution of radioactivity in the products of the enzymatic methylation of each of the labeled substrates. Histidine and its methyl and dimethyl derivatives were all readily converted to hercynine in the presence of the extract. No conversion of hercynine to ergothioneine was observed with this enzyme preparation. Table 1 also shows the suppressive effects of the presence of nonlabeled histidine and its methyl derivatives on the incorporation of radioactivity from the labeled substrates into the methylation products. The results obtained without added *S*-adenosylmethionine showed no significant methylation of histidine, methylhistidine, or dimethylhistidine.

DISCUSSION

The data in Table 1 demonstrate a significant conversion to hercynine of each of the labeled substrates in the presence of *S*-adenosylmethionine. Since a dialyzed enzyme preparation was used in these experiments, it contained no significant amounts of unlabeled, endogenous histidine or its methylated derivatives. The amounts of radioactivity appearing in the methylation products can therefore be used as a measure of the amounts of products formed during the incubation period. On this basis, it can be seen that methylhistidine and dimethylhistidine are methylated to a significantly greater extent than is histidine during the 6-hr incubation.

Undialyzed extracts of mycelium showed a greatly reduced ability to catalyze the methylation of the labeled substrates, compared to the results shown in Table 1 for the dialyzed extract.

This effect is ascribed to the presence of appreciable amounts of endogenous unlabeled histidine in the undialyzed extracts, with a resulting dilution of the radioactive label.

A suppressive effect of nonisotopic histidine, methylhistidine, and dimethylhistidine on the enzymatic methylation of the labeled substrates was observed with all three substrates. The effect was particularly evident when ^{14}C-histidine was the substrate; the presence of either methylhistidine or dimethylhistidine, at a concentration five times that of the ^{14}C-histidine, effectively prevented any significant methylation of the labeled substrate. This is explicable most simply on the basis of a preferential methylation of methylhistidine and dimethylhistidine compared to histidine. However, in these experiments it is not possible to distinguish between isotope dilution effects and true inhibitory effects, since nonisotopic substrates are present, and the extent to which these are methylated cannot, of course, be assessed by radioactivity measurements.

The presence of *S*-adenosylmethionine appears to be essential for each of the three methylation reactions. When the concentration of this methyl donor was made limiting, by the addition of only 0.02 μmole of the compound to the enzymatic incubation mixture, it was possible to attain a conversion of dimethylhistidine to hercynine equivalent to 64% of the *S*-adenosylmethionine present in the mixture. This suggests that the methyl-donating ability is due to *S*-adenosylmethionine itself rather than to other substances which may be present as impurities in the sample.

The present work with a cell-free extract confirms and extends studies carried out with growing mycelium of *N. crassa*. We have found that ^{14}C-labeled methylhistidine and dimethylhistidine, as well as histidine, are readily taken up by mycelium and are converted to hercynine and ergothioneine. Earlier work implicated the methyl group of methionine in the biosynthesis of ergothioneine (8) and, by means of double labeling of the methyl group of methionine with ^{14}C and deuterium, provided evidence to indicate that the conversion occurred by means of a triple transmethylation (10). The cell-free *N*-methyltransferase system reported here appears to be the first one described in which an amino acid is converted to its betaine by the successive addition of three methyl groups. Whether a single enzyme is involved in the triple methylation of histidine to form hercynine, or whether more than one is involved, as is the case in the biosynthesis of other trimethylammonium groups such as that of phosphatidylcholine (12), will require further studies with a purified enzyme system.

TABLE 1. *Methylation of histidine, methylhistidine, and dimethylhistidine by an enzyme preparation from Neurospora crassa*

Reaction mixture additions or omissions[a]	Distribution of radioactivity[b]			
	Histidine	Methylhistidine	Dimethylhistidine	Hercynine
^{14}C-His	56	0	0	44
^{14}C-His + MMH	100	0	0	0
^{14}C-His + DMH	100	0	0	0
^{14}C-His − SAM	100	0	0	0
^{14}C-His − enzyme[c]	100	0	0	0
^{14}C-His zero time	100	0	0	0
^{14}C-MMH	0	25	25	50
^{14}C-MMH + His	0	37	26	37
^{14}C-MMH + DMH	0	51	33	16
^{14}C-MMH − SAM	0	100	0	0
^{14}C-MMH − enzyme	0	100	0	0
^{14}C-MMH zero time	0	100	0	0
^{14}C-DMH	0	0	20	80
^{14}C-DMH + His	0	0	56	44
^{14}C-DMH + MMH	0	0	91	9
^{14}C-DMH − SAM	0	0	100	0
^{14}C-DMH − enzyme	0	0	100	0
^{14}C-DMH zero time	0	0	100	0

[a] Abbreviations: His, histidine; MMH, methylhistidine; DMH, dimethylhistidine; SAM, *S*-adenosylmethionine. The complete reaction mixture contained 0.6 μmole of *S*-adenosylmethionine, 0.2 μmole of substrate labeled uniformly with ^{14}C in the histidine moiety and with a radioactivity of approximately 24,000 counts per min, 1 μmole of unlabeled competitor, and 4 ml of enzyme preparation, in a total volume of 5 ml. Incubations were carried out at 25 C for 6 hr.

[b] Expressed as the percentage of the total radioactivity on the chromatogram. A value of 100% is equivalent to a radioactivity of 6,000 counts per min.

[c] Complete system containing enzyme extract which had been boiled for 5 min.

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