Biosynthetic Labeling of Diphthamide in *Saccharomyces cerevisiae*

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Diphthamide, the post-translational amino acid derivative in the diphtheria toxin-modification site of protein synthesis elongation factor 2 (EF-2), has the proposed structure 2-3-carboxyamido-3-(trimethylammonio)propyl]histidine (Van Ness, B. G., Howard, J. B., and Bodley, J. W. (1980) J. Biol. Chem. 255, 10710-10716). The identification of the biosynthetic precursors of diphthamide would provide a means of evaluating its proposed structure and determining if the amino acid occurs in proteins other than EF-2. To this end, yeast were grown on potential radiolabeled precursors and the resulting radiolabeled protein was hydrolyzed in acid. The acid hydrolysates were subjected to amino acid analysis with a program optimized to resolve diphthamine, the acid hydrolysis product of diphthamide, from the common amino acids. Radiolabel from [3H]histidine, [3H]methionine, and [Me-3H]methionine was found to be incorporated into diphthamine in a molar ratio of 1:1:3 while that of [35S]methionine was not incorporated. These results are in accord with the proposed structure of diphthamide and suggest that in its biosynthesis the backbone and 3 methyl groups of methionine are added to a histidine residue in the peptide chain of EF-2. These labeling experiments show that diphthamine (diphthamide) constitutes 6 x 10^-8 mol fraction of the total amino acids in yeast protein hydrolysates. Estimates of the amount of diphthamide present in the diphtheria toxin-modification site of EF-2 indicate that it constitutes from 4.5 to 9 x 10^-4 mol fraction of the total amino acids in yeast protein. The present evidence suggests that diphthamide occurs only in EF-2.

Investigation of the reaction mechanism of diphtheria toxin has revealed an unexpected structural complexity in its intracellular target, protein synthesis EF-2. At its site of ADP-ribosylation by diphtheria toxin, EF-2 contains an unusual amino acid (2-4) which we have named diphthamide (5). NMR analysis of diphthamide suggested that it is 2-3-carboxyamido-3-(trimethylammonio)propyl]histidine and led us to propose that its synthesis involves the post-translational modification of a histidine residue in EF-2 (6).

The present work was undertaken to test the proposed structure of diphthamide and to determine if diphthamide is unique to EF-2, through the identification of its biosynthetic precursors. Using specifically radiolabeled amino acids as precursors, we were able to observe and quantify radioactive incorporation into diphthamine, the hydrolysis product of diphthamide. The results of these biosynthetic studies are in complete agreement with the proposed structure and suggest that diphthamide does not occur in any protein other than EF-2.

**EXPERIMENTAL PROCEDURES**

**Materials**—L-[3H]Histidine (2.5 Ci/mmol) and L-[Me-3H]methionine (80 Ci/mmol) were obtained from Schwarz/Mann and New England Nuclear, respectively. L-[3H]methionine (81.1 Ci/mmol) and L-[35S]methionine (1220 Ci/mmol) were obtained from Amer sham. Radiolabeled amino acids were purified on the amino acid analyzer to remove low level contaminants prior to use in radioactive uptake experiments. Diphthine was prepared by hydrolysis of ribosyl-diphthamide (5) in 6 N HCl under vacuum at 110 °C for 24 h. Amino acid analyzer reagents were purchased from Pierce Chemical Co. All other common reagents were obtained from Sigma Chemical Co.

**Yeast Strains and Growth Conditions**—Two auxotrophic strains of *Saccharomyces cerevisiae* were employed in this study: EMS 63 (MATa, his2, gal1) from D. Livingston of this department and XJB3-1B (MATa, met6, gal2*) from the Yeast Genetic Stock Center, Berkeley, CA. Cells were maintained on a minimal growth medium consisting of 1.7 g of yeast nitrogen base (Difco) without amino acids or ammonium sulfate, 10 g of sucinic acid, 6 g of NaOH, 20 g of glucose, and 5 g of ammonium sulfate in 1 liter of water, supplemented with the required amino acid, L-histidine or L-methionine (40 mg/liter).

**Radioisotope Incorporation**—Incorporation experiments were conducted in minimal growth medium (50 ml) containing 150-700 pCi of radiolabeled amino acid and unlabeled amino acid to a final concentration of 2-3 mg/liter. Cultures of EMS 63 or XJB3-1B were inoculated with a midlog cell suspension (6% final inoculum) to initiate growth and were incubated in a shaking water bath at 29 °C for 16 or 22 h, respectively. The resulting midlog cell suspensions had a concentration of 1 x 10^8 cells/ml (A660 = 0.6-0.8). Radiolabeling experiments conducted with L-methionine in the methionine-compotent strain EMS 63 utilized carrier-free radioactive precursor.

For each culture, aliquots of the cell suspension (0.1 ml) were treated with 10% (w/v) trichloroacetic acid and the amount of radioactive incorporation into the precipitate was determined by liquid scintillation counting in a Beckman LS 7000, using Aquasol II. In the studies involving radiolabeled histidine, 30 ± 5% of the total radioactivity was incorporated into protein. When methionine was the radioactive precursor, 12 ± 5% of the total label was incorporated into protein. The same overall level of incorporation was observed for the biosynthetically competent strain (EMS 63) grown with carrier-free methionine.

The radioactive cells were harvested by centrifugation (6000 rpm x 10 min) and washed by centrifugation from unlabeled medium. Total protein was obtained by treating intact cells with 10% (w/v) trichloroacetic acid at 100 °C for 30 min. The precipitates were collected by centrifugation (15,000 rpm x 10 min), washed with water, centrifuged, and transferred to hydrolysis tubes using 0.5 ml of 6 N HCl. The tubes were sealed under vacuum and hydrolyzed for 72-96 h at 110 °C. These hydrolysates, which were derived from 50 ml of cell culture, contained approximately 35 mg of amino acids.

**Analysis of Radioactive Amino Acids**—A Beckman amino acid analyzer model 120C equipped with a long column (6 x 500 mm) and type AA-15 resin (22-μm particle size) was employed in the analysis.
Biosynthesis of Diphthamide

of the radioactive hydrolysates. Results with two slightly different programs are reported here. The three buffers used in the analysis of samples labeled with histidine (Fig. 1) were: first buffer (0.2 N trisodium citrate, 0.1 N NaCl, titrated to pH 4.12 with concentrated HCl); second buffer (0.35 N trisodium citrate, concentrated HCl to pH 5.26); third buffer (0.2 N trisodium citrate, 0.4 N NaCl, concentrated HCl to pH 6.40). The column was equilibrated with the first buffer and eluted with the second and third buffers at 70 and 120 min, respectively. The program used in the analysis of samples labeled with methionine (Fig. 2) contained: first buffer (as above); second buffer (0.38 N trisodium citrate, 0.02 N NaCl, concentrated HCl to pH 5.0); third buffer (0.2 N trisodium citrate, 0.8 N NaCl, concentrated HCl to pH 6.40). The column was equilibrated with the first buffer and eluted with the second and third buffers at 60 and 100 min, respectively.

Hydrolysates were dried under vacuum and dissolved in 0.05 M citric acid (250 µl) just prior to analysis. Initially, the analysis of radioactive samples (Fig. 1) was conducted with the aid of the ninhydrin detection system. In these cases, the column effluent (1.65 ml/min) was collected in 1- to 2-min fractions immediately after the colorimeter. Aliquots of these fractions were counted in Aquasol II in a Beckman LS-7500 scintillation counter programmed with Compton edge scattering to determine counting efficiency. Because the time delay between the colorimeter and the fraction collector was insig- nificant, the radioactive profiles were directly aligned with the recording of the ninhydrin color. Subsequently (Fig. 2) the ninhydrin-detection system was omitted. Under these conditions, the column effluent (1.2 ml/min) was collected in 1 to 2 fractions immediately after the column. Separate samples of the hydrolysate (2%) were analyzed to obtain ninhydrin profiles and these were aligned with the radioactive profiles by subtracting the time delay (8 min) between the fraction collector and the colorimeter. In all cases, the times reported in the figures, including the buffer changes, are in reference to the collected fractions. The maximum capacity of the amino acid analyzer under the conditions employed here is 6 mg of hydrolyzed protein.2 The reported analyses were conducted with a maximum of 4 mg of hydrolyzed protein.

The amount of radioactive diphthine eluting from the analyzer was determined by summing the radioactivity coincident with diphthine and subtracting a blank value. This blank value was established either by chromatographing a comparable level of unincorporated radioactive precursor (open circles, Figs. 1B and 2B) or by averaging the background level of radioactivity on either side of the radioactive diphthine peak (Fig. 2A). The fraction of precursor which was recovered as diphthine (Table I) was calculated by simply dividing the amount of radioactive diphthine by the amount of radioactivity recovered as the original precursor amino acid. For [β-3H]histidine, the conditions employed here is 6 mg of hydrolyzed protein.2 The reported analyses were conducted with a maximum of 4 mg of hydrolyzed protein.

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biosynthesis of diphthamide

recovery of incorporated radiolabel following hydrolysis and amino acid analysis

| Strain       | Precursor                  | Recovered label | Dpm diphthine | Calculated diphthine mole fraction |
|--------------|----------------------------|-----------------|---------------|----------------------------------|
|              |                            | Precursor | Diphthine | Dpm precursor | (× 10^5) | (× 10^5) |
| EMS 63 (his 2) | [β-3H]Histidine             | 522      | 148       | 3.1 ± 0.5   | 6.3     |           |
|              | [α-3H]Methionine            | 108      | 37.5      | 3.4         | 6.0     |           |
|              | [Me-3H]Methionine           | 72       | 75.2      | 10.4        | 6.1     |           |
| XJB3-1B (met 6) | [α-3H]Methionine           | 2.5 ± 0.3 |           |            |         | 4.4       |
|              | [Me-3H]Methionine           | 7.7 ± 0.4 |           |            |         | 4.5       |

* The data for histidine incorporation are from Fig. 1B and the data for methionine incorporation are from Fig. 2, A and B.

* The total amount of radiolabeled diphthine (dpm) minus a blank value (see “Experimental Procedures”) was divided by the total amount of label (dpm) recovered as either histidine or methionine. The result of the histidine incorporation experiment represents the average and standard deviation of 5 determinations. The result of [α-3H]methionine incorporation with XJB3-1B represents the average and standard deviation of 5 determinations. The result of [Me-3H]methionine incorporation with XJB3-1B represents the average and range of 2 determinations.

† For the purpose of these calculations, it was assumed that diphthine is assembled from β-3H]histidine, [α-3H]methionine, and [Me-3H]methionine in a mole ratio of 1:1:3. The diphthine fraction was calculated by multiplying dpm diphthine/dpm precursor times the precursor mole fraction. The mole fraction of histidine and methionine in yeast protein hydrolysates was 0.0213 and 0.0176, respectively. These values were determined by amino acid analyses and are the average of 8 determinations.

>99% of the incorporated radioactivity was recovered as histidine. For [α-3H]methionine, >95% of incorporated radioactivity was recovered as methionine while for [Me-3H] and [35S]methionine, 82-85% of incorporated radioactivity was recovered as methionine.

results and discussion

The structure of diphthamide (see Fig. 3), which we have proposed on the basis of its chemical properties, lead us to suggest that it arises from the post-translational modification of a histidine residue in EF-2 (6). We have previously shown that diphthamide is quantitatively converted to diphthine by acid hydrolysis (5) and that diphthine can be resolved from histidine and other common amino acids on the amino acid analyzer (3, 5). In the present study, we have grown a histidine auxotroph of yeast on [β-3H]histidine and subjected the resulting radiolabeled protein to complete acid hydrolysis and chromatography on the amino acid analyzer. By analyzing the chromatographic effluent, we sought to detect and quantify radiolabeled diphthine resulting from the post-translational modification of radiolabeled histidine.

The fraction of histidine one would expect to recover as radiolabeled diphthine can be calculated from the quantity of the two amino acids in yeast protein hydrolysates. The quantity of diphthine which would result from the hydrolysis of diphthamide in EF-2 can be estimated from the stoichiometry of the toxin-catalyzed ADP-ribosylation reaction. One mol of ADP-ribose is incorporated per mol of diphthamide (3). In this reaction, 4-8 × 10^-6 mol of ADP-ribose/100 g of yeast protein are incorporated (3, 5). Assuming that hydrolysis of 100 g of protein produces 0.9 mol of amino acids (Mf = 114), these values yield a mole fraction of 4.5-9 × 10^-4 for diphthine in the total amino acids of hydrolyzed yeast protein. Analysis of yeast protein hydrolysates showed that histidine constitutes 2.13 × 10^-2 mol fraction of total amino acids (Table I, footnote C). Therefore, the ratio of diphthine to histidine in yeast protein hydrolysates would be 2.1-4.2 × 10^-3. In long term labeling experiments, such as those employed here, radiolabeled histidine should be uniformly distributed among all histidine residues in protein. Thus, from 0.02 to 0.042% of total radiolabeled histidine in protein should be recovered as radiolabeled diphthine if diphthamide in EF-2 is formed from a histidine residue.

Fig. 1 shows a typical chromatographic ninhydrin profile of a yeast protein hydrolysate supplemented with purified diphthine. In this system, developed to maximize the separation of diphthine from histidine, the acidic and neutral amino acids elute partially resolved in the first isocratic buffer, the basic amino acids including histidine elute in the third isocratic buffer, and diphthine elutes alone (117.5 min) in the second isocratic buffer.

The open circles (right ordinate) in Fig. 1B describe the result of an experiment with radioactive histidine which was designed to test the resolving power of the chromatographic system described above. In this experiment, 2.64 × 10^4 dpm of [β-3H]histidine were added to unlabeled yeast protein and the mixture was subjected to acid hydrolysis and chromatography. Very little alteration of the labeled compound occurred during hydrolysis and [β-3H]histidine was well resolved from diphthine. There was not a peak of radioactivity in the region

B. Barrowclough, P. Dunlop and J. W. Bodley, unpublished observations.
of diphthine and the base-line radioactivity in this area, a total of 6500 dpm, 0.0025% of that applied, was well below the amount expected to result from the post-translational modification of histidine. Therefore, this chromatographic system should be capable of detecting diphthine labeling by radioactive histidine.

The closed circles (left ordinate) in Fig. 1B describe the result of an experiment in which yeast were grown in the presence of \(^{\beta\text{-H}}\)histidine and the radioactive protein was hydrolyzed and chromatographed. Only a single additional radioactive peak was observed in this chromatogram as compared to the control (Fig. 1B, open circles) and this peak was exactly coincident with the diphthine which had been added to the hydrolysate as carrier. This chromatographic coincidence between the carrier diphthine detected by the ninhydrin reaction and radioactivity was observed in several elution systems. Thus, histidine is a precursor of diphthine.

The quantitative relationship between radioactive histidine and diphthine observed in hydrolysates of yeast protein in this and other similar experiments is summarized in Table I (top row). The average value for the fraction of total radioactivity recovered as diphthine was 3.1 \(\times 10^{-4}\). Multiplying this value by the mole fraction of histidine in protein yields a mole fraction of diphthine in total protein of 6.3 \(\times 10^{-6}\). This value agrees very closely with the amount of amino acid calculated above which would result from the hydrolysis of diphthamide in EF-2. These results suggest that diphthamide occurs only in EF-2.

The proposed structure of diphthamide suggested methionine as the most likely source of additional carbon atoms in the post-translational modification of histidine (6). Methionine could be involved in two ways, by contributing its backbone and as a source of the 3 methyl groups. Labeling experiments with \([\alpha\text{-H}]\), \([\text{Me-}3\text{H}]\), and \([\text{S-}3\text{S}]\)methionine were undertaken to test these possibilities. The results of these experiments were more complex than those with histidine because methionine can undergo a variety of both chemical (7) and biosynthetic (8, 9) alterations. A number of labeled species were observed in hydrolysates of methionine-labeled protein as expected. We have not made a systematic effort to identify these derivatives. Fortunately, labeled methionine and most of its altered forms were well resolved from diphthine on the amino acid analyzer.

Typical analyses of methionine-labeled protein hydrolysates are shown in Fig. 2. Only the portion of the chromatogram in the vicinity of diphthine (101 min) is shown. With \([\text{Me-}3\text{H}]\)methionine as label, only a single radioactive species coincident with diphthine was observed in this region of the chromatogram (Fig. 2A). With \([\alpha\text{-H}]\)methionine as label, two radioactive species were seen (Fig. 2B). One of these (88 min) was present in the hydrolyzed sample of unincorporated \([\alpha\text{-H}]\)methionine (Fig. 2B, open circles) and was tentatively identified as homocysteine thiolactone by co-chromatography with the authentic compound (data not shown). The other labeled compound was chromatographically coincident with diphthine (Fig. 2B, closed circles) and was absent from the hydrolyzed sample of the unincorporated amino acid (Fig. 2B, open circles). \([\text{S-}3\text{S}]\)Methionine yielded two labeled species in this portion of the chromatogram, neither of which was coincident with diphthine. Thus, \([\text{Me-}3\text{H}]\) and \([\alpha\text{-H}]\)methionine but not \([\text{S-}3\text{S}]\)methionine are precursors of diphthine.

The results of the methionine-labeling experiments shown in Fig. 2, as well as others performed with the methionine anabolic auxotroph XJB3-1B, are summarized in Table I. With the methionine auxotroph, methionine can only be derived from the medium, thus precluding endogenous dilution of the radiolabel (10). The data show a ratio of incorporation of \([\alpha\text{-H}]\) to \([\text{Me-}3\text{H}]\)methionine into diphthine of 1:3 as predicted by the proposed structure of diphthamide (Fig. 2). This stoichiometry could only be observed if the radiolabeled forms of methionine were incorporated via pathways which do not require exchange of the radiolabel. The same stoichiometry was observed in the methionine-labeling experiments conducted with the histidine auxotroph EMS 63. Indeed labeling of EMS 63 with all three precursor amino acids resulted in an essentially identical calculated quantity of diphthine with overall stoichiometry of \([\beta\text{-H}]\)histidine, \([\alpha\text{-H}]\), and \([\text{Me-}3\text{H}]\)methionine of 1:1:3. As noted under "Experimental Procedures" the methionine auxotroph grew somewhat more slowly than the histidine auxotroph and would thus be expected to contain somewhat less EF-2 (11). In agreement with this observation, a slightly smaller quantity of diphthine was calculated from the methionine-labeling data with this strain (Table I). Thus, with both auxotrophic strains the fraction of radioactivity recovered as diphthine appears to be a direct reflection of the quantity of the amino acid present in protein hydrolysates.

The most straightforward interpretation of these incorporation experiments is that diphthamide is assembled from a histidine residue in EF-2 by the addition of the backbone of methionine and 3 methyl groups, thus entirely accounting for the organic components revealed by NMR analysis of diphthamide and its derivatives (6). Fig. 3 summarizes the relationship between the proposed structure of diphthamide and the radiolabeled precursors seen here to be incorporated into diphthine.

The present work does not define the sequence of reactions involved in the biosynthesis of diphthamide but there are two general ways in which the backbone of methionine could be added to an imidazole ring. One of these is the \(\gamma\)-replacement reaction which proceeds via a Schiff base intermediate (12). This possibility can be eliminated because it requires the exchange of the \(\alpha\)-proton of methionine which we have found incorporated into diphthine. The second mechanism is known to be involved in polyamine synthesis (13) and is presumed to be involved in the biosynthesis of the usual nucleoside 3-(3-carboxy-3-aminopropyl)uridine (14). This reaction involves a nucelophilic attack on the \(\gamma\)-methylene of S-adenosylmethionine or its decarboxylated derivative. In the present case, the nucleophile could be provided by an imidazolium 2-carbonan (15). S-Adenosylmethionine also appears to be the source of the three methyl groups in diphthamide because \([\text{Me-}3\text{H}]\)methionine appears to be incorporated into diphthine without significant dilution of the label. If this incorporation had proceeded via tetrahydrofolate, significant exchange of tritium would be expected (16).

Recently Moehring et al. (17) have described the properties of a set of animal culture cell mutants which exhibit in vitro resistance to diphtheria toxin. One type of mutant, Tox\(^+\), has properties which suggest that it results from a mutation in the EF-2 gene, perhaps in the codon which specifies the histidine that is converted to diphthamide. The second class of mutant, MOD\(^+\), has properties which suggest that the genes which are involved encode the post-translational enzymes that produce diphthamide. The MOD\(^+\) mutants comprise 3 complementation groups suggesting that at least 3 different proteins are required for the post-translational synthesis of diphthamide. Moehring et al. (17) found that one member of the class of MOD\(^+\) mutants is capable of acting in vitro to restore the toxin sensitivity of EF-2 and that this reaction requires a dialyzable factor. Recently, they have observed that the dialyzable factor can be replaced by \([\text{Me-}3\text{H}]\)S-adenosylmethionine with the concomitant incorporation of label into a protein which migrates with EF-2 on sodium dodecyl sulfate.
Biosynthesis of Diphthamide

gels. The properties of the toxin-resistant mutants described by Moehring et al. (17) are consistent with the in vivo labeling experiments described here.

The present work on the in vivo labeling of diphthamide, taken together with earlier work on its structure (6), suggests that a unique set of reactions exists for the post-translational synthesis of the amino acid from a single histidine residue in the diphtheria toxin-modification site of EF-2. The functional significance of these reactions, which apparently occur in all eucaryotic cells (4), is still obscure.

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