JOURNAL OF BACTERIOLOGY, Mar. 1983, p. 1342–1347
0021-9193/83/031342-06$02.00/0

Vol. 153, No. 3

Occurrence of Diphthamide in Archaebacteria

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Received 29 October 1982/Accepted 13 December 1982

We examined the nature of the diphtheria toxin fragment A recognition site in the protein synthesis translocating factor present in cell-free preparations from the archaebacteria *Thermoplasma acidophilum* and *Halobacterium halobium*. In agreement with earlier work (M. Kessel and F. Klink, Nature (London) **287**:250–251, 1980), we found that extracts from these organisms contain a protein factor which is a substrate for the ADP-ribosylation reaction catalyzed by diphtheria toxin fragment A. However, the rate of the reaction was approximately 1,000 times slower than that typically observed with eucaryotic elongation factor 2. We also demonstrated the presence of diphthine (the deamidated form of diphthamide, i.e., 2-[3-carboxyamide-3-(trimethylammonio)propyl]histidine) in acid hydrolysates of *H. halobium* protein in amounts comparable to those found in hydrolysates of similar preparations from eucaryotic cells (*Saccharomyces cerevisiae* and HeLa). Diphthine could not be detected in hydrolysates of protein from the eubacterium *Escherichia coli*. Whereas both archaebacterial and eucaryotic elongation factors contain diphthamide, they differ importantly in other respects.

Fragment A is an enzyme containing the 21,000-dalton N-terminal amino acid sequence of diphtheria toxin which, in the presence of NAD^+, blocks amino acid incorporation in eucaryotic cell-free extracts (4, 12). Inhibition of protein synthesis results from ADP-ribosylation of a single post-translationally modified histidine residue, diphthamide, present in elongation factor 2 (EF-2) of all plant and animal cells thus far examined (3, 16). Diphthamide does not appear to occur in eucaryotic proteins other than EF-2 (P. C. Dunlop and J. W. Bodley, J. Biol. Chem., in press). The corresponding elongation factor of eubacteria, EF-G, is not a substrate for fragment A, and amino acid incorporation in extracts of eubacteria is unaffected even by high concentrations of diphtheria toxin or its A fragment (4, 12).

Recently, however, Kessel and Klink (7, 8) have shown that amino acid incorporation in extracts of several species of archaebacteria is inhibited by diphtheria toxin in the presence of NAD^+ and that an elongation factor in these extracts is ADP-ribosylated. On the basis of these observations, they concluded that the archaebacterial factor is of the eucaryotic type. However, the concentrations of toxin used to inhibit incorporation in the archaebacterial extracts (between 1 and 10 μM) were extremely high compared with those needed to inhibit incorporation in extracts of eucaryotic cells. At such high concentrations, fragment A functions as a glycohydrolase and cleaves NAD^+ to nicotinamide and ADP-ribose (4, 12). Kun et al. (9) have reported that micromolar concentrations of ADP-ribose can slowly ADP-ribosylate certain proteins nonenzymatically at physiological pH.

Although archaebacteria resemble eubacteria in many ways, they possess certain other characteristics that, until recently, were thought to be found only in eucaryotes. For this reason, there is considerable interest in their significance in early evolution of microorganisms and in their possible ancestral relationship to primitive eucaryotes (5, 13, 18). It seemed important, therefore, to confirm the observations of Kessel and Klink and to determine whether the ADP-ribosylation of the archaebacterial elongation factor is, in fact, catalyzed directly by diphtherial fragment A and is not the result of a secondary reaction preceded by the hydrolysis of NAD^+. We also determined the diphthamide content of eubacterial and archaebacterial protein by analyzing their hydrolysates for radioactive diphthine after the incorporation of radioactive histidine. Diphthine was not detected in hydrolysates of eubacterial protein, but it was observed in archaebacterial hydrolysates in amounts comparable to those found in eucaryotic protein hydrolysates. Thus, whereas the archaebacterial elongation factor is ADP-ribosylated slowly by fragment A, the factor contains the post-translational modification which is important for recognition by fragment A.

MATERIALS AND METHODS

Cultures. *Halobacterium halobium* (NRC strain) was kindly sent to us by W. F. Doolittle. It was maintained at 42°C on a slight modification of the complex medium described by Bayley (1). The incorporation of [β-^{3}H]histidine into *H. halobium* was performed at 42°C in a synthetic medium in which an amino acid mixture without histidine replaced the yeast extract and tryptone of the complex medium. *Thermoplasma acidophilum* (strain 122-1B2) was generously supplied to us by D. G. Searcy of the University of Massachusetts as a frozen paste and was kept at −80°C until used. Commercial baker's yeast (Red Star Yeast Co.) was used to prepare EF-2. HeLa S3 cells (ATCC CCL 2.2) were grown at 37°C on Jokliks modified Eagle medium (GIBCO Laboratories) which contained 3.1 μg of histidine per ml and 5% newborn calf serum. *Escherichia coli* (*his*) was grown on minimal medium supplemented with 2.4 μg of histidine per ml.

Preparation of elongation factor-containing bacterial extracts. Frozen *T. acidophilum* (4.5 g) was ground with 5 g of alumina in the cold to a gummy consistency. Water was added, and the thick slurry was adjusted to pH 6.5 with 1 M Tris base and centrifuged for 15 min at 2,000 rpm. The turbid brown supernatant was withdrawn and the extraction was repeated several times. The pooled supernatants (10 to 11 ml) were then centrifuged for 35 min at 30,000 × *g*, and the supernatant was dialyzed in the cold against two 250-ml changes of 50 mM ammonium acetate–10 mM magnesium acetate–1 mM dithiothreitol. The dialyzed solution was then brought to 50% saturation with an equal volume of saturated ammonium sulfate. The precipitate was discarded, and the supernatant was brought to 75% saturation with ammonium sulfate. The precipitate, containing EF-2, was dissolved in 1.5 ml of 50 mM Tris-hydrochloride–0.5 mM dithiothreitol at pH 7.0 and desalted through Sephadex G-10 equilibrated in the same buffer. Portions of this solution, which was about 0.8 μM in EF-2, were stored at the temperature of liquid nitrogen until used. About 2.5% of the total protein was ADP-ribosylated in the presence of fragment A and excess NAD^+.

EF-2 from *Saccharomyces cerevisiae*. EF-2 was partially purified from baker's yeast as previously described (7). Further purification was achieved by chromatography on phenyl-Sepharose CL-4B by using gradient elution with 70% ethylene glycol. The resulting material was used in the present experiments. In the presence of fragment A and excess NAD^+, 1.4 nmol of ADP-ribose per mg of protein became trichloroacetic acid precipitable.

Fragment A. Fragment A was prepared from cross-reacting material 45 (2), and its thiol group was carboxymethylated to prevent dimerization. As previously shown, carboxymethylation has no effect on enzyme activity (12).

[^{32}P]ADP-ribose. Approximately 0.3 mg of carboxymethyl fragment A that had been stored under 80% saturated ammonium sulfate was centrifuged, the pellet was dissolved in 100 μl of 50 mM Tris buffer at pH 8, and 40 μl of freshly dissolved 0.5 mM NAD^+ and 20 μl (6 μCi) of [^{32}P]NAD^+ (ICN) were added. After 4 h at 37°C, 5 μl of 30 mM unlabeled ADP-ribose (Sigma Chemical) was added, and the mixture was passed through a Sephadex G-15 column equilibrated with 50 mM Tris at pH 8. Carboxymethyl fragment A emerged as a sharp peak with the void volume and was followed by a peak containing [^{32}P]ADP-ribose. The labeled ADP-ribose was shown by paper chromatography (butyric acid-ammonia-water, 66:1:33), and by its failure to ADP-ribosylate rabbit reticulocyte EF-2, to be free of all but traces of NAD^+.

Kinetics of ADP-ribosylation. The reaction mixture contained ~80 pmol of either *T. acidophilum* or *S. cerevisiae* EF-2, 140 μl of 50 mM Tris containing 0.1% bovine serum albumin at pH 7.1, and 40 μl of 100 μM [^{32}P]NAD^+ (2 to 3 μCi) in a total volume of 400 μl. The reaction mixture was incubated at 37°C, and ADP-ribosylation was initiated upon the addition of 20 μl of a suitable dilution of carboxymethyl fragment A. At intervals, 50-μl aliquots were withdrawn and quickly added to 1 ml of 5% trichloroacetic acid. The precipitates were collected on 3MM filters (Millipore Corp.), washed thoroughly with 5% trichloroacetic acid, dried, and counted in scintillation vials containing Omnifluor (New England Nuclear Corp.) plus toluene. In the case of *S. cerevisiae* EF-2, the buffer was 50 mM Tris at pH 7.6 containing 0.5% bovine serum albumin.

Incorporation of β-^{3}H]histidine. To minimize the incorporation of minor radiolabeled contaminants in [β-^{3}H]histidine (New England Nuclear Corp.), the radiolabeled amino acid was first purified by chromatography on an amino acid analyzer as described elsewhere (Dunlop and Bodley, in press). Cultures of *H. halobium*, HeLa S3, and *E. coli* (80, 100, and 50 ml, respectively) were grown as described above after the addition of 8 μCi of the purified [β-^{3}H]histidine per ml of culture.

At the conclusion of the incubation, the cells were harvested by centrifugation and suspended in 1 or 2 ml of water. The cellular protein was precipitated with 1/10 volume of 100% (wt/vol) trichloroacetic acid, and the mixture was heated for 15 min at 100°C. The precipitated protein was collected by centrifugation and washed several times in the centrifuge, first with 5% trichloroacetic acid and then with water. The washed precipitate was suspended in 0.8 ml of 6 N HCl and sealed under vacuum in a hydrolysis vial. The samples were hydrolyzed at 110°C for 72 to 96 h.

Quantitative determination of radiolabeled diphthine. The chromatographic resolution of histidine and diphthine present in the protein hydrolysates and the quantitative determination of radiolabel associated with each were accomplished by methods which are described in detail elsewhere (Dunlop and Bodley, in press). In brief, the radioactive protein hydrolysates were applied to a Beckman model 120C amino acid analyzer with type AA-15 resin and eluted with a series of three isocratic buffers which were selected to maximize the separation of diphthine and histidine. The analyzer effluent was collected in fractions, and these were analyzed by scintillation counting for their tritium content. The data were expressed as the fraction of total recovered radioactivity which was chromatographically coincident with diphthine.

RESULTS

Kinetics of ADP-ribosylation of EF-2 in cell-free extracts. We measured, as a function of

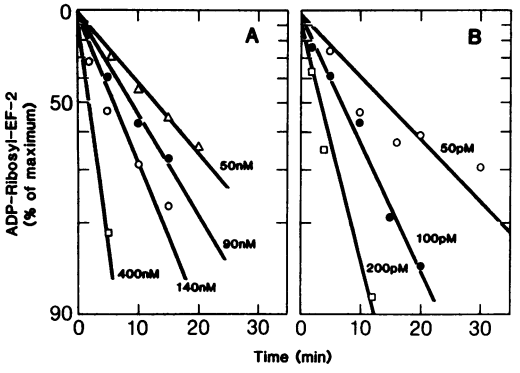

FIG. 1. Effect of increasing concentrations of fragment A on the rate of ADP-ribosylation of EF-2 from *T. acidophillum* (A) and *S. cerevisiae* (B). The fragment A concentrations used are indicated.

time, the [^{32}P]ADP-ribosyl-EF-2 produced in the presence of excess [^{32}P]NAD$^+$ (10 μM) and various concentrations of diphtherial fragment A with partially purified EF-2 preparations isolated from *T. acidophilum* and from *S. cerevisiae* (baker's yeast). The unlabeled EF-2 remaining at each time is expressed in Fig. 1A and B as the logarithm of the percentage of the initial EF-2 concentration. The EF-2 concentration in the preparations used was estimated from the total trichloroacetic acid-precipitable label observed after 15 min at 37°C in the presence of a large excess of fragment A, assuming the incorporation of one ADP-ribose molecule per molecule of EF-2. In both the archaebacterial an yeast reaction mixtures shown in Fig. 1, the initial concentration of EF-2 was approximately 0.4 μM.

From the data given in Fig. 1, turnover numbers (number of molecules of ADP-ribosyl-EF-2 formed per minute per molecule of fragment A) for archaebacterial and yeast EF-2s were calculated and are compared (Table 1) with the turnover numbers for rabbit reticulocyte EF-2 (6, 11) and the glycohydrolase activity (10) of fragment A. It is clear from Fig. 1 and Table 1 that under comparable conditions, the fragment A-catalyzed ADP-ribosylation of *T. acidophilum* EF-2 is about 3 orders of magnitude slower than that of *S. cerevisiae*, although it is still 10 to 20 times faster than that of the fragment A-catalyzed hydrolysis of NAD$^+$ to ADP ribose and nicotinamide.

The optimal temperature for growth of *T. acidophilum* is 59°C (13). The rate of the ADP-ribosylation reaction was not increased when the reaction was carried out at this temperature. Solutions of fragment A may be boiled for several minutes without a significant loss of enzymic activity when the temperature is brought back to 37°C (4, 12). We do not know, however, whether the enzyme retains its activity at the higher temperature.

Free ADP-ribose does not react with EF-2. To demonstrate that ADP-ribosylation was not a secondary reaction after NAD$^+$ hydrolysis, we incubated the archaebacterial EF-2 preparation with an excess of [^{32}P]ADP-ribose for 1 h at 37°C, both in the presence and in the absence of fragment A. There was no incorporation of label into trichloroacetic acid-precipitated protein. In other experiments, the archaebacterial preparation in a buffered reaction mixture containing 0.8 μM EF-2, 10 μCi of [^{32}P]NAD$^+$, 1.5 μM NAD$^+$, and 1.5 μM fragment A was incubated for 10 min at 37°C. The reaction was stopped, and portions were electrophoresed in 9% polyacrylamide slab gels overnight at 30 V and stained with Coomassie blue. After being destained and dried, the gels were autoradiographed. The apparent molecular weight of the major labeled component was 84,000, in close agreement with that reported for *T. acidophilum* by Kessel and Klink (7, 8). Under similar conditions, the major ADP-ribosylated protein in the yeast EF-2 preparation was somewhat larger, with an apparent molecular weight close to 95,000. Both the yeast and the archaebacterial autoradiographs showed several minor bands of lower molecular weight which we have presumed to be degradation products (15). When EF-2 preparations were incubated with excess [^{32}P]NAD$^+$ or [^{32}P]ADP-ribose in the absence of fragment A, no ADP-ribosylated bands could be detected by autoradiography, even after prolonged exposure.

We conclude that the ADP-ribosylation of archaebacterial EF-2 in the presence of fragment A is catalytic and that the reaction resembles that in typical eucaryotic extracts, albeit slower.

Analysis of diphthine in hydrolysates of archaebacterial protein. Elsewhere (Dunlop and Bodley, in press) we have described in detail a method for analyzing protein hydrolysates for diphthine (the deamidated form of diphthamide) as a means for assessing the quantity of diphthamide in cellular protein. These measurements

TABLE 1. Turnover number of fragment A with various ADP-ribose acceptors

Substrate	Turnover no. (mol of substrate/mol of fragment A per min)
Rabbit EF-2[a]	2,200
S. cerevisiae EF-2	600
T. acidophilum EF-2	0.5
Water[b]	0.05

[a] Data taken from reference 11.
[b] Data taken from reference 10.

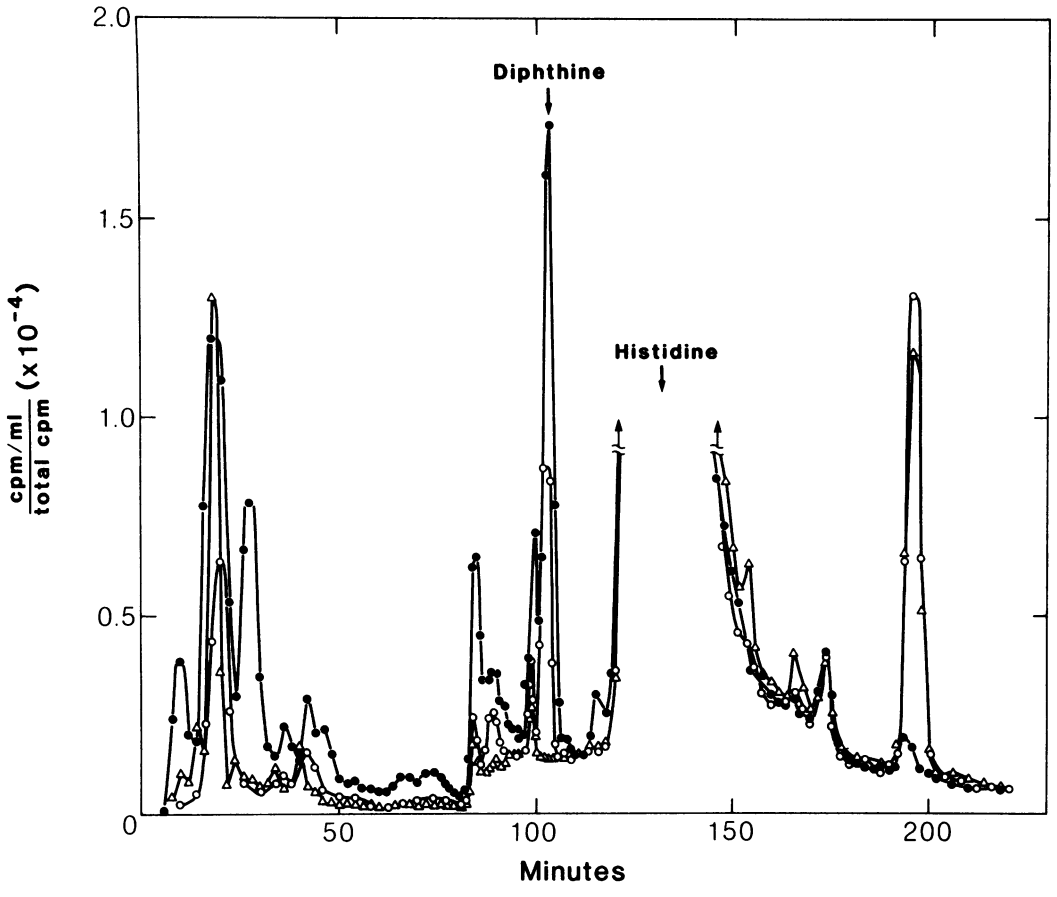

FIG. 2. Radioactive amino acid analyzer profiles of [β-^{3}H]histidine-labeled protein hydrolysates from *H. halobium* (●), HeLa cells (○), and *E. coli* (△).

suggested that in *S. cerevisiae*, diphthamide occurs only in the single toxin modification site of EF-2. The procedure uses the incorporation of radioactive histidine and is based on the fact that diphthamide in EF-2 is derived from the post-translational modification of histidine. Upon hydrolysis of total protein, diphthamide is converted to diphthine, and the fraction of radioactive histidine recovered in this form is a direct measure of the abundance of diphthamide.

Figure 2 shows the chromatographic profiles of radioactive protein hydrolysates derived from HeLa, *H. halobium*, and *E. coli* cells after each had been allowed to incorporate [β-^{3}H]histidine. The elution positions of diphthine and histidine, determined by the ninhydrin reaction, are designated in the figure. Radioactive diphthine was observed in the hydrolysates of HeLa and *H. halobium* protein but was undetectable in the hydrolysate of *E. coli* protein.

Table 2 summarizes the quantity of incorporated radioactivity which was recovered as diphthine in the various hydrolysates and compares these results with those we have previously reported for *S. cerevisiae*. A total of 0.024 to 0.05% of incorporated radioactivity was recovered as diphthine from the protein of each of the three cell types, i.e., archaebacterium, yeast, and human. Table 2 also shows the mole fraction of diphthine calculated from these radioactivity

TABLE 2. Abundance of diphthine based on incorporation of [β-^{3}H]histidine

Cell type	([^{3}H]diphthine/[^{3}H]histidine) × 10^{-4a}	(Diphthine/total amino acids) × 10^{-6b}
S. cerevisiae	3.3	6.3
HeLa	2.4	5.5
H. halobium	5.0	11.5
E. coli	<0.1	<0.1

[a] [^{3}H]diphthine and [^{3}H]histidine measured as number of disintegrations per minute.

[b] Diphthine and total amino acids measured in moles.

determinations and the abundance of histidine in the protein of the three cell types as determined by conventional amino acid analysis. A relatively constant value of 6 to 12 parts of diphthine per million parts of total amino acid was observed. An amino acid which occurs only once in a 1,000-residue protein such as EF-2 (which constitutes about 1% of the total cellular protein) would show an abundance of 10 ppm.

It is noteworthy that in the protein hydrolysates of these phylogenetically diverse cells there are few post-translational derivatives of histidine which are revealed by the type of analysis used in the experiments shown in Fig. 2. Independent experiments (data not shown) have demonstrated that the peak of radioactivity emerging at 18 min in all three chromatograms results from the chemical breakdown of [β-^{3}H]histidine during acid hydrolysis. In addition to this breakdown product, only two radioactive components, other than the peak of diphthine itself, were regularly seen. A radioactive component in hydrolysates of labeled *H. halobium* eluted at 28 min. Similarly, a radioactive species in hydrolysates of both *E. coli* and HeLa cell protein eluted at 196 min. The structure of these components is unknown, but they appear to be derived from histidine and to occur with an abundance of several parts per million.

DISCUSSION

In the present study we have confirmed the observation by Kessel and Klink (7, 8) that cell-free extracts of the archaebacterium *T. acidophilum* contain an 84,000-dalton elongation factor that becomes ADP-ribosylated in the presence of NAD^+ and relatively high concentrations of diphtherial fragment A. Moreover, diphthamide, thought of as the sine qua non of the toxin recognition site and hitherto found only in eucaryotic cells, has now been shown to be present in *H. halobium* in amounts equal to or greater than those present in typical eucaryotes. We have been unable to detect this amino acid in the protein of *E. coli*. The fact that diphthamide is present in *H. halobium* suggests that, at least in this respect, the archaebacterial elongation factor resembles the corresponding EF-2 in eucaryotes. However, as we have seen, the ADP-ribosylation reaction in archaebacteria proceeds at least 3 orders of magnitude more slowly. Because we experienced considerable difficulty in isolating active EF-2 preparations from the NRC strain of *H. halobium*, our kinetic studies were only carried out with *T. acidophilum* extracts. However, since Kessel and Klink (7) have already found that exceptionally high concentrations of toxin are required for the ADP-ribosylation of EF-2 from four unrelated archaebacterial species, including *Halobacterium cutirubrum* and *T. acidophilum*, and since the same requirement has already been independently confirmed for the two latter species by H. Y. Tsen and U. Rajbhandary (personal communication), it seems virtually certain that the low turnover number for fragment A that we observed using *T. acidophilum* extracts will also hold true for the other archaebacterial species. There are probably other differences as well. It has been reported, for example, that cycloheximide, a potent inhibitor of translocation in eucaryotes, has no effect on the *T. acidophilum* protein-synthesizing system (Tsen and Rajbhandary, cited in reference 13 and personal communication). We also note that the amino acid sequence in the neighborhood of the diphthamide residue has been highly conserved in species as diverse as yeasts, wheat, and cattle (14). But we have already argued (15) that features other than this conserved sequence itself are important to the recognition of the factor by fragment A. Peptides of EF-2 with M_r of <25,000, even though they contained diphthamide, failed to serve as substrates for the reaction. Thus, whereas archaebacterial EF-2 contains diphthamide and is acted on by diphtherial fragment A, only a detailed analysis of the factor will reveal the full extent of its structural and functional similarities to and differences from eucaryotic EF-2.

ACKNOWLEDGMENTS

This work was supported by National Science Foundation grant PCM-79-04914 (to A.M.P.) and by Public Health Service grant GM 26832 from the National Institutes of Health (to J.W.B.).

We thank D. G. Searcy of the University of Massachusetts for a generous gift of frozen *T. acidophilum* cells. We are also grateful to U. Rajbhandary for suggesting the use of *T. acidophilum* and who with H. Y. Tsen had shown in 1977 in unpublished experiments that a relatively high concentration of diphtheria toxin blocks translation in cell-free extracts of this organism although cycloheximide does not.

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