In Vitro Biosynthesis of Diphthamide, Studied with Mutant Chinese Hamster Ovary Cells Resistant to Diphtheria Toxin

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Diphthamide, a unique amino acid, is a post-translational derivative of histidine that exists in protein synthesis elongation factor 2 at the site of diphtheria toxin-catalyzed ADP-ribosylation of elongation factor 2. We investigated steps in the biosynthesis of diphthamide with mutants of Chinese hamster ovary cells that were altered in different steps of this complex post-translational modification. Biochemical evidence indicates that this modification requires a minimum of three steps, two of which we accomplished in vitro. We identified a methyltransferase activity that transfers methyl groups from S-adenosyl methionine to an unmethylated form of diphthine (the deamidated form of diphthamide), and we tentatively identified an ATP-dependent synthetase activity involved in the biosynthesis of diphthamide from diphthine. Our results are in accord with the proposed structure of diphthamide (B. G. VanNess, et al., *J. Biol. Chem.* **255**:10710-10716, 1980).

Protein synthesis elongation factor 2 (EF-2) possesses a unique amino acid, a derivative of histidine, with the proposed structure 2,3-[carboxyamido-3-(trimethylammonio)propyl]histidine. This amino acid has been given the trivial name diphthamide, and its acid hydrolysis product, from which the terminal amino group of the side chain has been cleaved, has been named diphthine (see Fig. 9) (13, 14). Diphtheria toxin inactivates EF-2 by catalyzing the covalent attachment of the ADP-ribose (ADPR) moiety of NAD⁺ to N-1 of the histidine imidazole ring of diphthamide (10), thereby inhibiting protein synthesis.

We have presented evidence, from studies of cultured mutant Chinese hamster ovary cells resistant to diphtheria toxin at the level of protein synthesis, that diphthamide is synthesized post-translationally (8). We isolated mutants with altered forms of EF-2 that resisted the action of diphtheria toxin both as intact cells and in ADP-ribosylation reactions catalyzed by the toxin in vitro. These mutants fall into two groups, based on their behavior in dominance hybridization studies. Those that behave codominantly are mutant in the structural gene that codes for EF-2. Those that behave recessively possess mutations which affect the post-translational biosynthesis of diphthamide, and we refer to them as modification, or MOD⁻, mutants. When EF-2 from one recessive MOD⁻ mutant, RPE.3b, was incubated with extracts of the EF-2 structural gene mutant RE1.22c, it was converted to an ADP-ribosylatable form. Extracts of the structural gene mutant were found to contribute both dialyzable and nondialyzable factors that were essential for modification of EF-2 to toxin sensitivity (8).

We now report the results of studies that characterize the in vitro biosynthesis of diphthamide further, using the two mutants previously described. We also identify MOD⁻ mutants that belong to two additional recessive complementation groups.

MATERIALS AND METHODS

Cells, media, and culture conditions. CHO-K1 Chinese hamster ovary cells, auxotrophic for proline (PRO), were

obtained from the American Type Culture Collection (CCL 61). Strain P1R2, prototrophic for proline and deficient in hypoxanthine-guanine phosphoribosyltransferase (HGPRT), was selected from CHO-K1 (6). Toxin-resistant mutants, altered at the level of EF-2, were selected as previously described (7) by exposure of mutagenized cells to either diphtheria toxin (strains RE1.22c, P1R2.DS, and P1R2.D17) or pseudomonas exotoxin A (strains RPE.3b, RPE.22e, and RPE.33d). Cells were maintained in Ham nutrient mixture F12 (GIBCO Laboratories, Grand Island, N.Y.) containing 5% fetal bovine serum in an atmosphere of 5% CO₂ in air.

Cell hybridization. Details of the hybridization procedure we employed in these studies have been presented previously (6). In each hybridization, one of the two parental strains was PRO⁻ HGPRT⁺ and the other PRO⁺ HGPRT⁻. All parental cells therefore could be eliminated by culture in proline-free hypoxanthine-aminopterin-thymidine medium (5) and the hybrid nature of recovered clones verified by their growth in this selective medium.

Preparation of cell extracts. Pelleted, washed cells were suspended in hypotonic lysing buffer (10 mM Tris-hydrochloride [pH 7.5], 10 mM KCl, 1.5 mM magnesium acetate, 6 mM 2-mercaptoethanol) at 10⁸ cells per 1.6 ml of buffer. Cells were allowed to swell and were then disrupted with a tight Dounce homogenizer, and 1.25 M sucrose was added to a final concentration of 0.25 M. The extract was centrifuged at 100,000 × g for 1 h and then dialyzed for 24 h against three changes of lysing buffer with 0.25 M sucrose. Extracts were stored under liquid nitrogen.

In vitro modification of EF-2. Unless otherwise noted, all extracts were used in reaction mixtures at a concentration of 1 mg of substrate (MOD⁻) extract protein and 1 mg of donor (structural gene mutant or other) extract protein per ml. Extracts might comprise up to 60% of the final volume of a reaction mixture. Unmixed extract controls were run for each time point and subtracted from the values for each mixed sample to derive specific counts. Reaction mixtures contained 30 mM Tris-hydrochloride (pH 7.5), 15 mM KCl, 5 mM magnesium acetate, and 6 mM 2-mercaptoethanol. S-Adenosyl-L-methionine (AdoMet) (chloride salt; Sigma Chemical Co., St. Louis, Mo.), radioactively labeled S-adenosyl-L-[methyl-¹⁴C]methionine (Ado[¹⁴C]Met) (Amer-

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sham Corp., Arlington Heights, Ill. or ICN Pharmaceuticals, Inc., Irvine, Calif.), or S-adenosyl-L-[methyl-³H]methionine (Ado[³H]Met) (Amersham) was added to a final concentration of 0.02 mM. In addition, an energy-generating system was included unless otherwise indicated: final concentrations of the usual complete system were ATP, 1 mM; GTP, 0.1 mM; CTP, 0.6 mM; creatine phosphate (CP), 10 mM; creatine phosphokinase (CPK), 80 µg/ml (all from Calbiochem, San Diego, Calif.). Reaction mixtures were incubated at 30°C. Some samples were precipitated with cold 10% trichloroacetic acid, collected on Whatman GF-C filter disks, washed extensively with 5% trichloroacetic acid, dried, and counted in Econofluor (New England Nuclear Corp., Boston, Mass.) in a Beckman LS7500 liquid scintillation spectrometer. Others were analyzed by toxin-catalyzed ADP-ribosylation (see below).

ADP-ribosylation of EF-2 modified in vitro. Reaction mixtures were assembled in an ice bath. Reaction mixtures contained 20% (vol/vol) of the modification mixtures described above. Each reaction mixture contained (final concentration) 34 mM Tris-hydrochloride (pH 7.5), 5 mM dithiothreitol, 3 mM magnesium acetate, 100 µM CaCl₂, 20 mM thymidine, 1.68 µM nicotinamide [*U*-¹⁴C]adenine dinucleotide (100 to 250 mCi/mmol; Amersham), and diphtheria toxin at 200 µg/ml (nicked, partially purified, 5.6 mg of toxin protein per ml; obtained from Connaught Medical Research Laboratories, Toronto, Ontario, Canada). Unless otherwise indicated, ADP-ribosylation mixtures were incubated for 15 min at 25°C and then precipitated with trichloroacetic acid, collected, and counted as described above.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Electrophoresis of proteins in 6, 7.5, and 15% (wt/vol) polyacrylamide slab gels containing 1% sodium dodecyl sulfate (SDS) was performed by the method of Laemmli (4). The ratio of the *N,N'*-methylenebisacrylamide (BioRad Laboratories, Richmond, Calif.) to acrylamide (Kodak, Rochester, N.Y.) was 0.8:30. Stacking gels were 3% acrylamide for 6 to 7.5% separating gels and 3.5% acrylamide for 15% separating gels. Protein bands were stained with Coomassie brilliant blue. Phosphorylase *b* (Sigma) was used as a marker protein for EF-2.

Fluorography of radioactively labeled proteins in gels. Destained gels were prepared for fluorography by the method of Chamberlain (1), dried, and exposed to Kodak X-Omat AR-5 film at -70°C.

Preparation and analysis of tryptic peptides from EF-2. Preparations containing radioactively labeled EF-2 were fractionated on 6% SDS-polyacrylamide gels and then stained, destained, and soaked in distilled water for 30 min. EF-2 bands were excised and completely dried in siliconized tubes at 50°C. Tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin (Worthington Diagnostics, Freehold, N.J.), 50 µg/ml in 0.1 M NH₄HCO₃ (pH 8), was added in the amount of 420 µl per gel slice, and slices were incubated at 37°C for 16 h. Supernatants were collected, dried in a vacuum centrifuge (Savant Instruments, Inc., Hicksville, N.Y.), and stored at -20°C until use. For analysis on SDS-polyacrylamide slab gels, dried peptides were solubilized in Laemmli sample buffer (4) and fractionated on 15% acrylamide gels. After a brief (15-min) staining and destaining, gels were dried and fluorographed as described above. For analysis on isoelectric-focusing gels, dried peptides were solubilized without boiling in O'Farrell lysis buffer A (9) and focused in glass tubes as described by O'Farrell (9). Gels were expressed from the tubes and cut into 2-mm slices. Each slice was placed in a 7-ml scintillation vial, in 1 ml of

NCS solubilizer-water (9:1) (Amersham), and incubated for 1 to 2 h at 60°C. Glacial acetic acid (20 µl) and Econofluor (6 ml; New England Nuclear) were added, and the radioactivity was determined in a scintillation spectrometer. To determine the pH gradient, gel slices were eluted in 0.5 ml of boiled distilled water for 1 to 3 h, and the pH was measured. Molecular weight determinations of radioactively labeled peptides were made with a Sephadex G-50 fine column (65 by 1.6 cm; Pharmacia Fine Chemicals, Piscataway, N.J.). The column was equilibrated and eluted with 0.1 M sodium phosphate buffer (pH 7.2), and the following six molecular weight markers, with their molecular weights indicated, were used (vitamin B₁₂, 1,350; insulin A chain, 2,300; bovine trypsin inhibitor, 6,150; cytochrome *c*, 12,800; myoglobin, 17,000; α-chymotrypsinogen, 25,700). UV absorbance at 280 nm was continuously monitored, fractions were collected, and radioactivity was determined by scintillation counting in Aquasol 2 (New England Nuclear).

Metabolic labeling of EF-2 with [³H]histidine. Subconfluent monolayers of CHO-K1 cells were incubated for 24 h in Eagle minimal essential medium, with nonessential amino acids, but prepared without histidine so that [*G*-³H]L-histidine (ICN) could be added to a concentration of 0.17 mg/liter (22 µCi/ml). Dialyzed fetal bovine serum (5%) was also added. Cells were harvested and extracts prepared as described above.

Amino acid analysis of protein hydrolysates. For amino acid analysis of [³H]histidine-labeled EF-2, extracts of labeled cells were concentrated in a collodion bag protein concentrator (Schleicher & Schuell, Inc., Keene, N.H.) and fractionated on 6% SDS-polyacrylamide gels as described above. Marker lanes of phosphorylase *b* (Sigma) were stained with Coomassie brilliant blue and used to cut out the unstained EF-2 bands. EF-2 was eluted into 50 mM Tris-hydrochloride (pH 7.5) with 0.3 M sucrose-0.1 M KCl-0.1 mM EDTA-5 mM dithiothreitol, concentrated again, and hydrolyzed under vacuum in 6 N HCl at 110°C for 48 to 60 h. For analysis of ¹⁴CH₃-labeled diphthamide modified in vitro, reaction mixtures were precipitated with 10% trichloroacetic acid, centrifuged at 9,000 × *g*, and washed twice with distilled water. Precipitates were hydrolyzed as described above. Samples were evaporated to dryness on a vacuum centrifuge (Savant), rehydrated in water and raised to pH 11 with 5 N KOH to remove ammonia, dried again, and then rehydrated and reduced to pH 1 with 6 N HCl. A final drying preceded rehydration in 0.2 N sodium citrate buffer (pH 2.2). Samples were analyzed on a Beckman model 121 amino acid analyzer with a single 50-cm long column (AA-15 resin) and by a modified three-buffer methodology (3) (Table 1). Analyzer effluent was collected, and radioactive counts were determined by scintillation counting in Aquasol 2 (New England Nuclear).

TABLE 1. Modified program for amino acid analysis of protein hydrolysates

Buffer	pH	Sodium citrate concn (N)	NaCl concn (N)	Buffer change time (min) ^a
A	4.12	0.2	0.1	60
B	5.00	0.38	0.02	100
C	6.40	0.2	0.8	

^a Column equilibrated with buffer A; buffer change times measured from time of sample injection.

TABLE 2. Mutant cell strain characterization

Analysis	Cell strains	Recessive CG (MOD ⁻ strains)	Phenotype ^a	% inhibition ^b	ADP-ribosylatable EF-2 (pmol/mg) ^c
A. Cell strains studied	CHO-K1c		Sensitive, wild-type	97–100	91–110
	P1R2		Sensitive, wild-type	97–100	103–109
	RPE.3b	CG-1	Resistant, recessive	0–5	0
	P1R2.DS	CG-1	Resistant, recessive	0–4	0
	P1R2.D17	CG-2	Resistant, recessive	3–7	2–7
	RPE.33d	CG-3	Resistant, recessive	0–3	1–3
	RPE.22e	CG-3	Resistant, recessive	0–5	0–3
	RE1.22c		Resistant, codominant	0–5	0–4
B. Dominance hybridization	RPE.3b × P1R2			95–100	105–119
	P1R2.DS × CHO-K1c			98–100	ND ^d
	P1R2.D17 × CHO-K1c			97–100	88–96
	PRE.33d × P1R2			96–100	89–91
	RPE.22e × P1R2			96–100	ND
	RE1.22c × P1R2			41–55	57–65
C. Complementation	RPE.3b × P1R2.DS			0–7	ND
	RPE.3b × P1R2.D17			97–100	90–98
	P1R2.DS × RPE.33d			92–100	ND
	RPE.3b × RPE.22e			90–95	87–90
	P1R2.D17 × RPE.22e			92–100	ND
	P1R2.D17 × RPE.33d			94–99	88–92
	RPE.33d × RPE.22e			0–9	0–3
	RE1.22c × RPE.3b			40–52	46–54
	RE1.22c × P1R2.D17			25–70	30–38
	RE1.22c × RPE.33d			25–61	ND

^a The phenotype, with respect to toxin sensitivity of EF-2, was determined by dominance hybridization of resistant mutants with cells possessing wild-type EF-2. A minimum of 4 and a maximum of 10 hybrids were screened in each case.

^b Percent inhibition of protein synthesis after a 24-h exposure to a saturating concentration of diphtheria toxin; 0.82 nM for sensitive cells, or 820 nM in the case of the resistant strains. A saturating concentration of toxin is that concentration which will cause maximum inhibition in minimum exposure time.

^c Picomoles of ADPR bound per milligram of cell extract protein in the toxin-catalyzed reaction.

^d ND, Not determined.

RESULTS

Characterization of mutant cell strains. The cell strains used in these studies are presented in Table 2 (part A) and characterized with respect to their sensitivity to diphtheria toxin and their content of toxin-sensitive ADP-ribosylatable EF-2. The results of dominance hybridization tests involving the toxin-resistant strains and strains with normal EF-2 are presented in Table 2 (part B). By submitting the toxin-resistant strains to complementation analysis (Table 2 [part C]) we were able to assign the five recessive MOD⁻-resistant strains to one of the three complementation groups (CGs; CG-1, CG-2, and CG-3) designated in Table 2 (part A).

In vitro modification of EF-2. We have previously reported that the non-ADP-ribosylatable EF-2 in cell extracts of MOD⁻ strain RPE.3b can be converted to toxin sensitivity in vitro by incubating it in a modification reaction with extracts of the EF-2 structural gene mutant RE1.22c before reacting it with toxin and NAD⁺ in a standard ADP-ribosylation mixture (8). This conversion was dependent on both dialyzable and nondialyzable factors present in the RE1.22c extract and also required the addition of an exogenous energy source.

When dialyzed extracts were used, the MOD⁻ EF-2 could be converted to an ADP-ribosylatable form only with the addition of amino acids to the reaction mixture. The essential amino acid was determined to be methionine (Fig. 1). Since in vivo methionine is converted to AdoMet in the

FIG. 1. Conversion of toxin-resistant EF-2 from CG-1 strain RPE.3b to ADP-ribosylatability in vitro. Dialyzed extracts of RPE.3b and structural gene mutant RE1.22c were used in a reaction mixture containing an energy system. Samples were withdrawn at indicated times, reacted in diphtheria toxin-catalyzed ADP-ribosylation assays, and picomoles of [¹⁴C]NAD⁺ bound per milligram of RPE.3b protein was determined. Symbols: Δ , no amino acids (AA) added; $\square$, 160 μ M methionine (Met) added; $\circ$, 16 μ M AdoMet added.

presence of ATP, we next substituted AdoMet for methionine. Under these conditions, conversion of MOD⁻ EF-2 to toxin sensitivity proceeded at an accelerated rate, and essentially all of the EF-2 from either mutant in MOD⁻ CG-1 was converted to toxin sensitivity. AdoMet was, therefore, included as a component of the standard reaction mixture in subsequent experiments.

Dialyzed extracts of cells belonging to MOD⁻ CG-2 and CG-3 were also active in catalyzing the modification of EF-2 from cells in CG-1. However, unlike the results obtained through cell hybridization, the EF-2 from mutants in CG-2 (P1R2.D17) or CG-3 (RPE.22e) were not converted to toxin sensitivity upon incubation with extracts of either RPE.3b or RE1.22c cells (Fig. 2).

AdoMet-mediated methylation of EF-2. AdoMet is an important methyl donor in transmethylation reactions. To determine whether AdoMet was functioning as a methyl donor in the in vitro modification of EF-2, we incubated extracts of RPE.3b and RE1.22c in reaction mixtures with Ado[¹⁴C₃]Met. ¹⁴CH₃ was incorporated into the acid-precipitable fraction of each of these extracts at a linear rate when the extracts were incubated unmixed (data not shown). However, when these extracts were adjusted to contain an equal content of cell protein, mixed, and incubated, a proportionately greater incorporation of ¹⁴CH₃ occurred. By deducting the picomoles of ¹⁴CH₃ incorporated by equal amounts of protein in unmixed cell extract, it was apparent that the incorporation which occurred only in the combined extracts proceeded at a rapid rate and was complete by 120 to 180 min (Fig. 3a). No increased, specific incorporation of ¹⁴CH₃ into extracts of CG-2 or CG-3 cells could be demonstrated when these extracts were combined with extracts from any of the mutants or from wild-type cells (data not shown).

The conversion of EF-2 to ADP-ribosylatability proceed-

FIG. 2. Cell-free modification reaction with extracts from cells of various diphtheria toxin-resistant groups. Extracts were reacted in complete reaction mixtures and samples withdrawn at indicated times for assay by toxin-catalyzed ADP-ribosylation. Symbols: □, RPE.3b (CG-1) + RE1.22c (gene mutant); ●, RPE.3b + P1R2.D17 (CG-2); ○, RPE.3b + RPE.22e (CG-3); ▲, P1R2.D17 + RE1.22c; △, RPE.22e + RE1.22c.

FIG. 3. Effect of the presence of an energy system on incorporation of ¹⁴CH₃ and conversion to ADP-ribosylatability of EF-2 from RPE.3b cells. Extract from gene mutant RE1.22c was used as methyltransferase donor. (a) Incorporation of ¹⁴CH₃ from AdoMet in the presence and absence of the energy system (ES). (b) Conversion of RPE.3b EF-2 to ADP-ribosylatability in the presence and absence of the energy system. The reaction mixture without AdoMet contained the energy system.

ed at a slower rate than methylation and was generally complete by 360 min. Omission of the energy system from the modification reaction had no effect on the rate of incorporation of ¹⁴CH₃ or the total amount incorporated into acid-precipitable material. However, there appeared to be less ADP-ribosylatable EF-2 formed in the absence of an exogenous energy source, as only 16 to 30% as much [¹⁴C]ADPR was incorporated per milligram of cell protein under standard assay conditions (Fig. 3b).

Further analysis of EF-2 modified in the presence and absence of an energy system revealed that the difference was not in the amount of resistant EF-2 that could be converted to ADP-ribosylatability, but rather in the rate at which the modified EF-2 could be ADP-ribosylated after methylation. In Fig. 4, the kinetics of toxin-catalyzed covalent binding of ADPR to normal EF-2 from wild-type cells is compared with the binding of ADPR to EF-2 modified in the presence and absence of an energy system. EF-2 modified in the presence of an energy system was ADP-ribosylated at the same rate as EF-2 from wild-type cells. EF-2 modified in the absence of an energy system bound as much ADPR per milligram of protein but did so at a reduced rate.

Methylation of RPE.3b extract protein was complete ca. 4 h before the maximum conversion of RPE.3b EF-2 to an ADP-ribosylatable form was reached. This suggested that methylation preceded the energy-dependent step. We confirmed this first by incubating mixtures of RPE.3b and

FIG. 4. Rate of ADP-ribosylation of EF-2 from RPE.3b cells modified in vitro compared with rate of ADP-ribosylation of wild-type EF-2. Cell extract from RE1.22c was used as methyltransferase donor. Modification reaction mixtures were incubated for 360 min and then samples containing 50 μ g of RPE.3b protein were incubated in ADP-ribosylation assays for the indicated times. An extract of CHO-K1 cells provided wild-type EF-2, which was assayed at 36 μ g of cell protein per sample. Symbols: $\circ$, CHO-K1 extract; $\blacktriangle$, RPE.3b extract, modified in the presence of an energy system; $\bullet$, RPE.3b extract, modified with no energy system.

RE1.22c extracts in reaction mixtures that were complete, except for AdoMet, and then by adding AdoMet and monitoring incorporation of $^{14}\text{CH}_3$ and conversion to ADP-ribosylatability. If the energy-dependent step proceeded in the absence of methylation, then the rate of conversion to ADP-ribosylatability should have approximated the rate of methylation after the addition of AdoMet. The rates of methylation and conversion to ADP-ribosylatability did not change after this preincubation, however (data not shown).

Thus, the modification in vitro of EF-2 from RPE.3b cells occurs in two steps. First, there is the methylation of EF-2 which is mediated by AdoMet and does not require an additional energy source; then, there is a second, energy-dependent step. After methylation, EF-2 can be ADP-ribosylated, but only at a reduced rate. After the second, energy-dependent step, the modified EF-2 can be ADP-ribosylated at approximately the same rate as EF-2 extracted from wild-type cells.

Determination of essential component of the energy system.

We determined that ATP was the essential component of the energy system by testing each nucleoside triphosphate alone in modification reaction mixtures (Table 3). ATP at the concentration normally used (1 mM), plus CP and CPK, was as efficient as the complete system in converting EF-2 from RPE.3b to a form that could be ADP-ribosylated as rapidly as wild-type EF-2. If the concentration of ATP was raised to 3 mM, CP and CPK were not necessary. Neither GTP nor CTP, alone or in combination with CP and CPK, could substitute for ATP. Concentrations of 5 mM or more of these compounds caused precipitation and loss of activity in the reaction mixtures. In current experiments we omit GTP and CTP.

TABLE 3. Effect of various components of the energy system on conversion of RPE.3b EF-2 to ADP-ribosylatability^a

ATP (mM)	GTP (mM)	CTP (mM)	CP (mM) ^b	CPK (μ g/ml) ^b	% of complete system
1	0.1	0.6	10	80	100
—	—	—	—	—	18
1	—	—	—	—	25
3	—	—	—	—	109
1	—	—	10	80	117
3	—	—	10	80	94
—	1	—	—	—	19
—	3	—	—	—	14
—	1	—	10	80	17
—	3	—	10	80	19
—	—	1	—	—	20
—	—	3	—	—	11
—	—	1	10	80	24
—	—	3	10	80	5

^a In vitro modification mixtures contained RPE.3b and RE1.22c extracts in the standard mixture with components of the energy system varied. The first line represents the complete system. Modification mixtures were incubated for 360 min at 30°C, and ADP-ribosylation mixtures were incubated for 15 min at 25°C. A total of 41 pmol of [^{14}C]ADPR was incorporated per mg of RPE.3b protein that reacted with the complete system.

^b —, Not used.

Characterization of methylated proteins. Radiolabeled proteins in extracts incubated with Ado[^{14}C]Met were separated by electrophoresis on polyacrylamide gels and analyzed by fluorography (Fig. 5). Extracts of EF-2 structural gene mutant RE1.22c and recessive MOD[−] mutant RPE.3b were incubated separately and as mixtures in the presence of [^{14}C]Met. In each case, fluorography revealed the same pattern of minor $^{14}\text{CH}_3$ -labeled bands, presumably the results of exchange reactions catalyzed by endogenous meth-

FIG. 5. Comparison of radioactively labeled proteins in cell extracts from modification mixtures and wild-type CHO-K1 cells. Proteins were separated by SDS-polyacrylamide gel electrophoresis. Radioactive proteins were detected by fluorography. Extracts in lanes 1, 2, and 3 were incubated in modification mixtures with Ado[^{14}C]Met. Lane 1, gene mutant RE1.22c alone; lane 2, MOD[−] mutant RPE.3b alone; lane 3, RE1.22c + RPE.3b; lane 4, RE1.22c + RPE.3b extracts modified with unlabeled AdoMet then ADP-ribosylated with [^{14}C]NAD⁺; lane 5, extract of wild-type CHO-K1 cells ADP-ribosylated with [^{14}C]NAD⁺.

yltransferases present in each extract. Incubation of the two extracts together produced a single additional strongly labeled band with an M_r of 94,000. This protein coelectrophoresed with ADP-ribosyl-EF-2 from wild-type CHO-K1 cells and with the protein that was ADP-ribosylated by diphtheria toxin after *in vitro* modification of toxin-resistant EF-2 in the presence of unlabeled AdoMet. In double-label experiments, modification was carried out in the presence of Ado[^3H]Met, followed by ADP-ribosylation with [^{14}C]NAD $^+$. In gel electrophoresis, the two radiolabels migrated as a single band which, when cut out and counted, gave a CH $_3$ -ADPR molar ratio of 3.1:1.

Analysis of tryptic peptides by polyacrylamide gel electrophoresis. To determine whether the ADPR and methyl groups were incorporated into the same tryptic peptide, we analyzed tryptic digests of EF-2 by electrophoresis on 15% SDS-polyacrylamide gels. [^{14}C]ADP-ribosyl EF-2 from wild-type CHO-K1 cells yielded a single radiolabeled peptide band with an apparent M_r of 3,600 (Fig. 6). [^{14}C]ADPR-labeled peptides derived from rat liver and mouse L-cell EF-2 also migrated to this position (data not shown). When a tryptic digest of RPE.3b EF-2 that was modified in the presence of Ado[^3H]Met and then ADP-ribosylated with [^{14}C]NAD $^+$ was analyzed, all of the radiolabel was recovered in a single band that coelectrophoresed with the radiolabeled peptide derived from wild-type EF-2 (Fig. 6, lane 2). The CH $_3$ -ADPR molar ratio in each of these bands was 3.2:1, which is in agreement with the ratio determined by analysis of double-labeled EF-2 recovered from polyacrylamide gels.

In some experiments, a second, more anodal band with an apparent M_r of 1,800 was detected by fluorography and by cutting out and counting gel slices (Fig. 6, lane 3). This second band comigrated with the radiolabeled band present in tryptic digests of ADP-ribosyl EF-2 from RPE.3b cells, when the latter was modified in the absence of an energy-generating system (Fig. 6, lane 4). The CH $_3$ -ADPR molar ratio in both of the bands seen in Fig. 6, lane 3, was 3.1:1. These results suggest that a precursor relationship exists between the more anodal band and the band that comigrated with the ADP-ribosyl peptide found in tryptic digests of EF-2 extracted from CHO-K1 cells.

The rate of migration of low-molecular-weight peptides in

FIG. 6. Comparison of peptides from wild-type CHO-K1 EF-2 and EF-2 modified *in vitro*. Peptides were prepared from labeled EF-2, separated on a 15% SDS-polyacrylamide gel, and detected by fluorography. Lane 1, CHO-K1 EF-2 ADP-ribosylated with [^{14}C]NAD $^+$; lane 2, MOD $^-$ mutant RPE.3b EF-2 modified in the presence of gene mutant RE1.22c extract, an energy system, and Ado[^3H]Met (360 min) and then ADP-ribosylated with [^{14}C]NAD $^+$ (30 min); lane 3, RPE.3b EF-2 modified and ADP-ribosylated as described in lane 2 but with a different lot of RE1.22c extract (incomplete modification assumed); lane 4, RPE.3b EF-2 modified as described in lane 2 but with no energy system; lane 5, RPE.3b EF-2 modified in the presence of RE1.22c extract, an energy system, and Ado[^{14}C]Met (no ADP-ribosylation); lane 6, RPE.3b EF-2 modified as described in lane 5 but with no energy system.

SDS-polyacrylamide gels can be influenced by charge as well as by size (2, 12). Differences in the rates of migration of the labeled peptides seen in Fig. 6 could, therefore, be due to differences in net charge rather than to differences in molecular size. Indeed, when tryptic digests of modified RPE.3b EF-2 were subjected to gel filtration on a Sephadex G-50 column, the two peptides seen by gel electrophoresis could not be resolved. All of the radioactivity eluted as a single symmetrical peak corresponding to a molecular weight of 3,730. Similar elution profiles were obtained with tryptic digests of EF-2 isolated from rat liver and CHO-K1 cells. When the three tryptic digests were combined and chromatographed, all of the radioactivity again eluted in a single peak (M_r , 3,750).

These results suggest that the peptides have similar or identical sizes but differ in net charge. The net charge of each peptide observed on electrophoresis gels could depend on whether the modification was carried out in the presence or absence of an energy system, as well as the presence or absence of a covalently bound ADP-ribosyl group. To investigate the charge properties of the radiolabeled peptides further, we subjected them to analysis on electrofocusing gels.

Electrofocusing patterns of tryptic peptides. When EF-2 was modified with Ado[^{14}C]Met in the absence of an energy source, a single radiolabeled tryptic peptide, with an apparent pI of 7.2, was obtained (Fig. 7a). Modification of EF-2 in the presence of an energy source produced a charge alteration that shifted the peptide in the basic direction (pI, 7.4) (Fig. 7e). When modification was carried out in the presence of an energy source, frequently all of the EF-2 was not converted to the form that yielded the more basic peptide, and both radiolabeled peptides could be recovered from the gel (Fig. 7c).

The isoelectric points of the two corresponding ADP-ribosyl peptides were shifted toward the acid pH (i.e., they had lower isoelectric points). Although more widely separated on the gel, the ADP-ribosyl peptides were in the same relative positions as the underivatized peptides. The ADP-ribosyl form of the peptide, with a pI of 7.2, focused at pH 4.2, whereas the addition of ADPR to the more basic peptide shifted its pI to 5.2 (Fig. 7b and f). The corresponding ADP-ribosyl peptide obtained by tryptic digestion of EF-2 from CHO-K1 cells also had a pI of 5.2 (Fig. 7g), suggesting that it too would focus at pH 7.4 in its underivatized form.

After toxin-catalyzed ADP-ribosylation of the RPE.3b EF-2 that was modified *in vitro*, essentially all of the methyl label in the tryptic digests was shifted toward the anode. Thus, the toxin-catalyzed ADP-ribosylation of RPE.3b EF-2 *in vitro* is an efficient reaction that converts virtually all of the modified EF-2 to ADP-ribosyl EF-2.

Analysis of radioactive amino acids. It was clear from electrophoretic analysis of the products of modification that the methyl groups donated by AdoMet were incorporated into EF-2. Precisely where was suggested to us by the structure of diphthamide proposed by Bodley and his colleagues (3, 13) (see Fig. 9). It seemed likely that modification of EF-2 from RPE.3b involved transfer of methyl groups from AdoMet to an amino group at the C-3 position of the aliphatic side chain, thus converting it to the trimethylamine. Since diphtheria toxin catalyzes transfer of a single ADPR to the histidine imidazole ring of diphthamide, this interpretation is also consistent with the CH $_3$ -ADPR molar ratio of 3.1:1 obtained after ADP-ribosylation of the modified RPE.3b EF-2.

To verify that this was indeed the case, we hydrolyzed the

FIG. 7. Isoelectric focusing of tryptic peptides and ADP-ribosyl peptides from wild-type EF-2 and EF-2 modified in vitro. (a through f), Focusing of EF-2 peptides from MOD⁻ strain RPE.3b modified in the presence of gene mutant RE1.22c extract. (a) No energy system + Ado[¹⁴C]Met; (b) no energy system + AdoMet + [¹⁴C]NAD⁺; (c) energy system + Ado[¹⁴C]Met (incomplete modification); (d) energy system + AdoMet + [¹⁴C]NAD⁺ (incomplete modification); (e) energy system + Ado[¹⁴C]Met; (f) energy system + AdoMet + [¹⁴C]NAD⁺; (g) CHO-K1 EF-2 ADP-ribosylated with [¹⁴C]NAD⁺; (h) rat liver EF-2 ADP-ribosylated with [¹⁴C]NAD⁺.

modified EF-2 and analyzed it by ion-exchange chromatography on an amino acid analyzer. We compared the methylated residues in RPE.3b EF-2 with purified diphthine from *Saccharomyces cerevisiae* and with diphthine in EF-2 obtained from CHO-K1 cells grown in medium containing [³H]histidine. The ninhydrin elution profile of a standard amino acid mixture, to which purified yeast diphthine was added, is presented in Fig. 8a. Since all of the samples were first hydrolyzed in acid, which converts diphthamide to its deamidated hydrolysis product diphthine (14), the peak at 118 min corresponds to diphthine. When EF-2, purified from parental CHO-K1 cells that were metabolically labeled with [³H]histidine, was hydrolyzed and the products analyzed, there was a radiolabeled peak at the position of yeast diphthine (Fig. 8b). This suggests that EF-2 from CHO-K1 cells possesses diphthamide with the same properties as yeast diphthamide. When mutant cells representing the three MOD⁻ CGs were similarly labeled and analyzed, none had a typical diphthine peak, indicating that all were impaired in their ability to synthesize diphthamide (data not shown). When EF-2 from RPE.3b cells, modified in the presence of Ado[¹⁴C]Met and an energy system, was hydrolyzed and analyzed, it gave the profile shown in Fig. 8c. Again, a methyl-labeled residue coeluted with yeast diphthine. When extracts of the MOD⁻ mutant RPE.3b and structural gene mutant RE1.22c were incubated separately in the modification reaction mixture and then mixed before hydrolysis, no radiolabeled peak was detected in the region of diphthine. The only significant difference in the profiles was found at the position of diphthine. Omission of energy from the modification mixture gave an elution profile identical to that shown in Fig. 8c.

DISCUSSION

From CHO-K1 cells, we isolated MOD⁻ mutants that belong to three distinct CGs. By metabolic labeling with [³H]histidine and analysis of hydrolyzed amino acids, we

determined that none of these mutants possess EF-2 containing normal diphthamide (unpublished data). Our experimental evidence indicates that the diphthamide residue in the EF-2 of cells of these three CGs may lack different portions of the four-carbon side chain because of mutations affecting enzymes that catalyze post-translational modifications of EF-2 and steps in the synthesis of diphthamide. When exposed to normal modification enzymes in cell-cell hybrids, the biosynthesis of diphthamide is completed. For cells of CG-2 and CG-3, the conditions of living cell hybrids have, thus far, been required to complete the modification. For EF-2 from cells of the remaining group, CG-1, we were able to establish conditions to complete the modification in vitro.

Under these conditions, we showed that AdoMet mediates transfer of methyl groups to an intermediate form of diphthamide in the EF-2 of CG-1 cells. It is this step in the biosynthesis of diphthamide that appears to be blocked in the mutants of CG-1. The methylation step is dependent on a heat-labile, nondialyzable cytosolic factor(s) which is present in extracts of wild-type cells, EF-2 structural gene mutants, and mutants of the two other recessive MOD⁻ CGs, CG-2 and CG-3. This factor appears to be a methyltransferase enzyme that is responsible for transferring three methyl groups from AdoMet to the amino group of the diphthamide side chain. Alternatively, it is also possible that more than one transferase is required for this step, and cells of CG-1 lack the enzyme that transfers at least the first methyl group to the amino group. After methylation in the absence of ATP, EF-2 can be ADP-ribosylated, but at a reduced rate compared with the rate of ADP-ribosylation of EF-2 from wild-type cells. A second modification step, which is dependent on methylation and requires ATP, converts the EF-2 to a form that is ADP-ribosylated at approximately the same rate as EF-2 from wild-type cells. The data indicate that this step may be amidation of the terminal carboxyl group on the aliphatic side chain of diphthine, catalyzed by a "diphthamide synthetase."

FIG. 8. Demonstration of $^{14}\text{CH}_3$ -labeled diphthine by amino acid analysis of protein hydrolysate. (a) Ninhydrin elution profile showing position of yeast diphthine. (b) Radioactive profile of hydrolysate of $[^3\text{H}]$ histidine-labeled EF-2 from wild-type CHO-K1 cells. (c) Radioactive profile of hydrolysate of mixed, in vitro-modified extracts of MOD^- strain RPE.3b and gene mutant strain RE1.22c reacted with $\text{Ado}[^{14}\text{C}]\text{Met}$. (d) Radioactive profile of hydrolysate of extracts of MOD^- strain RPE.3b and gene mutant strain RE1.22c incubated separately in reaction mixtures containing $\text{Ado}[^{14}\text{C}]\text{Met}$ and then mixed immediately before hydrolysis.

To explain our results, we propose that RPE.3b possesses EF-2 with the intermediate form of diphthamide shown in Fig. 9. In the absence of ATP, AdoMet donates three methyl groups to form the trimethylamine and converts the interme-

diate to diphthine. In the presence of ATP, an amide is then formed from the carboxyl group of the side chain. This converts diphthine to diphthamide and reduces the negative charge on the side chain. This model is consistent with the ionic properties of the methylated tryptic peptides and ADP-ribosyl peptides on isoelectro-focusing gels. The amidation of diphthine increases the net positive charge of the modified side chain and produces a shift in the tryptic peptide toward the cathode. Addition of an ADP-ribosyl group, with a net charge of 2^- , then shifts the peptide toward the anode. Since acid hydrolysis of diphthamide cleaves the amide and yields the free acid, diphthine (2-[3-(carboxy)-3-(trimethylammonio)propyl]histidine), this model also explains why, after acid hydrolysis of the modified EF-2, the methylated products formed in the presence and absence of an energy source were chromatographically coincident on the amino acid analyzer.

If the reaction sequence proposed in our model is correct, then there may be four distinct enzymatic steps in the biosynthesis of diphthamide. Two of these steps may involve the addition of the four-carbon side chain to the imidazole ring of histidine. Dunlop and Bodley have recently shown that this side chain is contributed by methionine in *Saccharomyces cerevisiae* (3). The EF-2 from neither CG-2 nor CG-3 mutants can be methylated or modified to ADP-ribosylatability in vitro under conditions appropriate for modification of CG-1 EF-2, although each possesses methyltransferase activity. CG-2 and CG-3 mutants can, however, complement each other in cell-cell hybrids. This evidence, and evidence from amino acid analysis of metabolically labeled EF-2 from these mutants (unpublished data), indicates that their EF-2 does not possess the side chain of diphthamide. Therefore, mutants in each of the two CGs may be deficient in a different enzyme activity required for the addition of the backbone of methionine to the imidazole ring of histidine. Alternatively, failure to add methionine to the histidine imidazole ring in CG-2 or CG-3 mutants could result either from failure to modify a specific site, on EF-2, which recognizes the modification enzyme or failure of the modification enzyme itself to undergo a required post-translational modification.

The third enzymatic step, the one lacking in CG-1 mutants, involves addition of methyl groups to form the trimethylammonio group. This may be carried out by one, or more than one, transferase. However, it is clear from the CH_3 -ADPR molar ratio that CG-1 mutants are blocked at the addition of the first methyl group. Dunlop and Bodley (3) have shown that methionine contributes three methyl groups

FIG. 9. Proposed structure of an intermediate form of diphthamide, 2-(3-carboxy-3-aminopropyl)histidine, in the EF-2 of CG-1 mutants and its sequential conversion to diphthamide in vitro.

to diphthamide in the peptide chain of yeast EF-2. Our findings are in full accord with theirs and, furthermore, support their proposed structure of diphthamide. The fourth step, we believe, involves the amidation of the carboxyl group of diphthine to form diphthamide. This step requires ATP and is dependent upon the methylation step. If it were not, it would have occurred *in vivo* in CG-1 mutants.

Under our experimental conditions, the methyltransferase activity lacking in CG-1 mutants is specific for a single soluble cytosolic protein, EF-2. This agrees with the conclusion of Dunlop and Bodley (3) that diphthamide occurs only in EF-2. We have not, however, ruled out the possibility that other proteins removed in our fractionation procedure might also be methylated by this enzyme. Further experiments will determine this. Whether the other modification enzymes also have the same specificity remains to be determined, but indications are that the enzyme system for the biosynthesis of diphthamide is highly specific.

According to our findings, EF-2 can be ADP-ribosylated by diphtheria toxin when either diphthine or diphthamide is present in the peptide chain. However, when diphthine is present, ADP-ribosylation proceeds at a slower rate. Pappenheimer et al. (11) have recently demonstrated that strains of the unique archaebacteria contain EF-2 that is ADP-ribosylatable, although they require much higher concentrations of diphtheria toxin than normal eucaryotic EF-2. They have demonstrated that archaebacterial EF-2 does contain diphthine, but their method of analysis, involving acid hydrolysis, did not permit determination of diphthamide. It is possible that diphthine, and not diphthamide, is normally present in archaebacteria, and this contributes to the slow ADP-ribosylation of their EF-2. Such a finding would undoubtedly have evolutionary significance. Furthermore, if stable intermediate forms of diphthamide occur in the peptide chain of eucaryotic EF-2 *in vivo*, they might play a role in regulating the biosynthesis or activity of EF-2, either directly or by modulating the rate and extent of its ADP-ribosylation by an endogenous ADP-ribosyl transferase.

It is clear that EF-2 undergoes a remarkable series of related post-translational modifications, involving first the biosynthesis of the unique amino acid residue diphthamide and then its ADP-ribosylation with the covalent attachment of the ADP-ribosyl moiety to the N-1 of the imidazole ring. The physiological significance of these modifications, which appear to occur in all eucaryotes and even the archaebacteria, remains an enigma.

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