Selective in Situ Labeling of Mitochondrial Proteins by 2′-Bromo-2,4-dinitroacetanilide†

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ABSTRACT: Bovine heart mitochondria and phosphorylating electron transport particles were labeled with 2′-bromo-2,4-dinitroacetanilide-^{14}C. Subsequent sodium dodecyl sulfate gel electrophoresis in each case showed only two radioactive polypeptide bands of approximate molecular weights 44,000 and 31,000, respectively. This type of selective in situ labeling inhibits oxidative phosphorylation in mitochondria and ATP-driven reduction of NAD^+ by succinate in phosphorylating electron-transport particles. It was found that the rate of phosphorylation of ADP coupled to the oxidation of malate in mitochondria was reduced by a factor of 2 when only 0.4 mol of the label/mol of cytochrome aa_3 was covalently bound to mitochondrial membrane. The major labeled protein was isolated and found to exhibit a single subunit protein band of approximate molecular weight 44,000 in dodecyl sulfate gel electrophoresis. Except for the cysteine residues which react directly with the labeling reagent, the labeled protein has within experimental error the same amino acid composition as the coupling factor F_B isolated and characterized as described in a separate report. The close similarity in amino acid composition and the equality of subunit molecular weights suggest that the radioactive protein was probably produced by in situ alkylation of F_B by this reagent.

The in situ labeling of mitochondrial proteins by 2,4-dinitro-5-(bromo[2′-^{14}C]acetoxyethoxy)phenol (DNBP)[1] and its effect on oxidative phosphorylation were reported recently (Wang et al., 1973; Copeland et al., 1974). Subsequently, through the syntheses and studies of a large number of uncoupler derivatives, we found that the compound 2′-bromo-2,4-dinitroacetanilide (BDNA) to be an even better in situ alkylating reagent for mitochondrial proteins. The sodium dodecyl sulfate gel electrophoresis pattern of bovine heart mitochondria labeled by 2′-bromo-2,4-dinitro[2′-^{14}C]acetanilide showed essentially only two radioactive bands. Labeling by BDNA, which is practically complete in a few minutes at room temperature, suppresses state 3 respiration and oxidative phosphorylation in mitochondria as well as ATP-driven reduction of NAD^+ by succinate in ETP. Apparently an optimum balance between the affinity of the labeling reagent for the hydrophobic coupling sites and the reactivity of its leaving group is required for selective labeling. For example, we found that 2,4-dinitrophenylbromo[2′-^{14}C]acetate effectively suppresses state 3 respiration, but labels mitochondrial proteins almost nondiscriminatively. On the other hand, we found that *N*-bromoacetyl-3′-nitroanthranilanilide ($O_2NC_6H_4NH$-CO-$C_6H_4NHCOCH_2Br$) has no effect on the state 3 respiration of mitochondria.

The results of the labeling experiments with BDNA, the concurrent measurements of oxidative phosphorylation and related enzymatic activities, and the isolation and identification of the principal BDNA-labeled protein in mitochondria are reported in the present article.

Our general strategy is to exploit the specificity of local membrane structures by labeling in situ the macromolecules and their functional groups which participate in the coupling of electron transport to phosphorylation, subsequently pull the labeled molecules out of the membrane for identification, and then use the so identified molecules and their functional groups as a guide in exploring the primary energy transduction process in mitochondria and chloroplasts.

Materials and Methods

Chemicals. 2′-Bromo-2,4-dinitroacetanilide (BDNA). 2,4-Dinitroanaline (5 g, 27 mmol) was suspended in 20 ml of benzene in a 100-ml round-bottom flask fitted with a magnetic stirring bar and reflux condenser. To this suspension was added bromoacetyl bromide (8.266 g, 41 mmol) dropwise over 2–3 min. The reflux condenser was then protected with a $CaSO_4$ drying tube and the mixture refluxed for 14 hr. The reaction started to evolve HBr after 20 min at reflux. At the end of the reflux period the solution was cooled and the benzene and excess bromoacetyl bromide were removed by rotary evaporation under reduced pressure (aspirator vacuum ≈20 mm). The residue was an amber oil which crystallized when washed and triturated with cyclohexane. The crude crystals were filtered and recrystallized from benzene–cyclohexane (1:1) as yellow crystals, mp 97–100°. The reaction gives the amide in 84% yield (7 g).

This material was further recrystallized several times from benzene–cyclohexane–ethyl acetate (10:10:1) to give beautiful white needles, mp 99.5–100.5°. This material gave only one spot on thin-layer chromatography (TLC) (silica gel–20% ethyl acetate–benzene) with an R_f of 0.7 while in the same system, 2,4-dinitroaniline had an R_f of 0.4. Anal. Calcd percentages for $C_8H_6N_3O_5Br$: C, 31.58; H, 1.97; N, 13.82. Found: C, 31.52; H, 2.11; N, 13.58.

2′-Bromo-2,4-dinitro[2′-^{14}C]acetanilide (BDNA-^{14}C).

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[1] Abbreviations used are: DNBP, 2,4-dinitro-5-(bromo[2′-^{14}C]acetoxyethoxy)phenol; BDNA, 2′-bromo-2,4-dinitroacetanilide; cyc *c*, cytochrome *c*; HBHM, heavy bovine heart mitochondria; RCR, respiratory control ratio; ETP, electron-transport particles.

[2-^{14}C]Bromoacetic acid (specific activity 3.074 Ci/mol) was converted to 2-bromoacetyl chloride with thionyl chloride in benzene. 2,4-Dinitroaniline was then added and the mixture heated at 80° for 14 hr. The crude material was transferred to a preparative TLC plate and purified by chromatography (benzene). The radioactive band was removed and recrystallized (benzene–cyclohexane–ethyl acetate, 10:10:1) using Craig tube technique to give a crystalline product, mp 99.5–100.5°. The radioactive amide was obtained in 54% yield.

[2-^{14}C]Bromoacetic acid was obtained from New England Nuclear Corp. ^{32}P-labeled phosphoric acid (carrier free in 0.02 *M* HCl solution) was from New England Nuclear Corp. ADP disodium salt (Grade I), ATP disodium salt (crystalline, from equine muscle), bovine serum albumin (crystallized and lyophilized), cytochrome *c* (Type IV), and 5,5′-dithiobis(2-nitrobenzoic acid) were purchased from Sigma Chemical Co. Bovine serum albumin solutions (generally 200 mg/ml) were dialyzed against at least six changes of distilled water, adjusted to pH 7.4, and made 0.25 *M* with respect to sucrose before use.

Bovine Heart Mitochondria (BHM). The mitochondria were prepared essentially as described by Löw and Vallin (1963). Fresh bovine hearts were obtained from a local slaughterhouse and transported under packed ice. After the removal of connective tissue and fat, the hearts were cut into small cubes, minced, and suspended in a 0.25 *M* sucrose solution at 0°. The pH was then adjusted to 7.4 with Tris and the mixture subsequently filtered through cheesecloth. The mince was resuspended in an ice-cold 0.25 *M* sucrose + 15 m*M* EDTA solution (pH 7.4) and the pH again checked and adjusted to 7.4 with Tris. The suspension was homogenized for 50 sec with a Tekmar-Tissumizer (Tekmar Co., Cincinnati, Ohio). The supernatant obtained after centrifuging the homogenate at 800*g* for 20 min was again centrifuged at 12,000*g* for 20 min. The resulting pellet was suspended in a cold preparation medium containing 0.25 *M* sucrose and 10 m*M* Tris buffer at pH 7.4, and again homogenized with a Potter-Elvehjem glass–Teflon homogenizer. The mixture was centrifuged for 15 min at 17,000*g*. Heavy mitochondria (HBHM) in the sediment were separated and resuspended in the preparation medium and homogenized. The homogenate was centrifuged at 17,000*g* for 15 min. Heavy mitochondria were separated, resuspended in the preparation medium, and homogenized to give 40–60 mg of protein/ml.

Electron-Transport Particles. The preparation of these particles has been described by Hansen and Smith (1964).

Chemical Modifications. Modified HBHM were prepared by incubating freshly prepared HBHM with BDNA at 23° for 10 min in a medium containing 0.25 *M* sucrose, 20 m*M* Tris, 10 m*M* potassium chloride, 2 m*M* magnesium chloride, and 5 m*M* phosphate at pH 7.4, and 3–5 mg of mitochondrial protein/ml. The mitochondria were spun down (10 min at 17,000*g*) and the unbound BDNA was removed with the supernatant. The pellet was washed by gentle suspension in 0.2 ml of bovine serum albumin solution which contained 25 mg of bovine serum albumin and 0.25 *M* sucrose at pH 7.4. After 5 min the suspension was diluted with 10 ml of 10 m*M* Tris and 0.25 *M* sucrose buffer at pH 7.4, and centrifuged for 10 min at 17,000*g*. The supernatant was siphoned out and the pellet was washed with the preparation medium and centrifuged at 4°. The final pellet of BDNA-labeled, bovine serum albumin washed BHM was resuspended in 1.0 ml of the same buffer at a concentration of about 25 mg of protein/ml.

Modified ETP were prepared by incubating ETP with BDNA at 23° for 10 min in a medium containing 0.25 *M* sucrose and 0.01 *M* Tris buffer at pH 7.5 and 3–5 mg of protein/ml. The ETP were spun down (40 min at 177,000*g*) and the unbound BDNA was removed with the supernatant. The pellet was homogenized in the sucrose–Tris buffer at pH 7.5 at a concentration of about 15–20 mg/ml.

Assay of Coupled Phosphorylation and Oxidation. The experimental procedures are the same as described in previous reports (Wang et al., 1973; Copeland et al., 1974). All assays were performed at 23°. The energy-linked reduction of NAD^+ by succinate was measured according to the method of Ernster and Lee (1967).

Assay of the Redox Efficiency of Electron Carriers. NADH–cytochrome *c* reductase and succinate–cytochrome *c* reductase activities in ETP were measured spectrophotometrically at 23°, by following the reduction of cytochrome *c* at 550 nm (Sottocasa et al., 1967). Cytochrome *c* oxidase activity was measured spectrophotometrically by following the change in absorbance at 550 nm due to the oxidation of reduced cytochrome *c*. The rates were recorded as soon as the substrates, NADH, succinate, and reduced cytochrome *c*, were added into the prepared medium containing ETP. The enzymatic activities of the dehydrogenases were measured spectrophotometrically by following the changes in absorbance at 340 nm. The rate of dehydrogenation of β-hydroxybutyrate was recorded as 15 μmol of β-hydroxybutyrate was injected into the 3-ml solution containing 0.1 m*M* NAD^+, 3 μ*M* rotenone, 0.2–0.5 mg of ETP, and 50 m*M* phosphate buffer at pH 7.5 (Gotterer, 1967). For assaying the activity of malate dehydrogenase, 15 μmol of oxaloacetate was injected into 3 ml of phosphate buffer containing 0.2 m*M* NADH, 3 μ*M* rotenone, and 0.1–0.3 mg of supernatant protein (see footnote *b* of Table III) at pH 7.5.

Dodecyl Sulfate Gel Electrophoresis. Polyacrylamide gels (10%, 10 mm or 6 mm diameter, 100 mm length) were used for dodecyl sulfate gel electrophoresis according to the method of Weber and Osborn (1969). Dansylated bovine serum albumin (mol wt 68,000), carbonic anhydrase (mol wt 31,000), and ribonuclease (mol wt 13,700) were added to gels for the determination of the approximate molecular weight of the labeled subunit polypeptides. The gels were cut with Gilson gel fractioner and put into plastic counting vials. To each vial were added 1.5 ml of distilled water and 5 ml of Aquasol (New England Nuclear Corporation). After each mixture was shaken, the sample appeared uniformly suspended. The radioactivity in each vial was assayed with a Beckman LS-233 scintillation counter.

Isolation of the BDNA-Labeled Protein. Freshly prepared HBHM (1.4 g) was suspended in 0.25 *M* sucrose and 0.01 *M* Tris buffer at pH 7.4 at a protein concentration of 5 mg/ml. Radioactive BDNA was added to a final concentration 3×10^{-5} *M*. The solution was incubated at room temperature for 10 min; fourfold volume of cold acetone was then added to the solution. The pellet obtained by centrifugation of the sample at 27,000*g* for 10 min was washed with 80% cold acetone and centrifuged again. The pellet from the second centrifugation, modified HBHM acetone powder, was kept for the next step.

Modified HBHM acetone powder was extracted by homogenization with 0.1 *M* Tris-chloride buffer at pH 8.8 containing 0.5 m*M* dithiothreitol at a protein concentration of 50 mg/ml. After standing overnight, the suspension was centrifuged at 45,000*g* for 10 min. The supernatant was

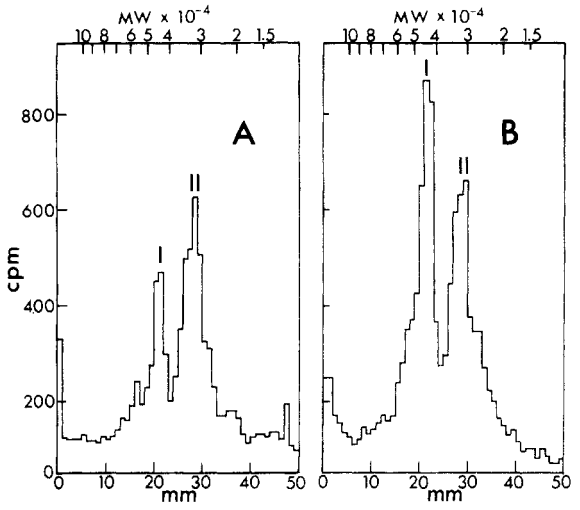


FIGURE 1: Dodecyl sulfate gel electrophoresis patterns of BDNA-^{14}C-labeled polypeptides in phosphorylating ETP (A) and bovine heart mitochondria (B), respectively. Incubation time = 10 min. Concentration of BDNA = 3×10^{-5} M. Each logarithmic molecular weight scale at the top of the diagrams was determined by the best linear semilogarithmic plot determined by the dansylated standard proteins.

fractionated with ammonium sulfate. The precipitates formed between 25 and 60% saturated ammonium sulfate were collected and redissolved in 10 mM Tris-sulfate buffer containing 0.1 mM dithiothreitol at pH 8.0. The solution was passed through a Bio-Gel P-2 column (10 mm × 600 mm) and eluted with 10 mM Tris-sulfate buffer containing 0.1 mM dithiothreitol at pH 8.0 to remove the remaining ammonium sulfate. The concentrated protein fractions were added into a Whatman DE-52 column (10 mm × 160 mm) which had been preequilibrated with 10 mM Tris-sulfate and 0.1 mM dithiothreitol at pH 8.0. Elution of the adsorbed protein was monitored by absorbance at 280 nm. The fractions of the most radioactive peak eluted with 10 mM Tris-sulfate buffer containing 0.1 mM dithiothreitol at pH 8.0 were combined and ammonium sulfate was added (350 mg/ml of eluate to give approximately 60% saturation). The precipitates from the ammonium sulfate precipitation were collected and redissolved in 2.5 mM Tris-sulfate buffer containing 0.1 mM dithiothreitol at pH 8.0 and passed through another Bio-Gel P-2 column (10 mm × 160 mm) to remove the excess salts. The new fractions containing the most radioactive protein peak were allowed to stand at 5° for 2 hr. The solution became cloudy and was centrifuged. The pellet was collected which contained about 0.3 mg of pure labeled protein. The supernatant was kept for further precipitation (which yielded less pure labeled protein).

Amino Acid Analysis. The pure labeled protein was hydrolyzed in 6 N hydrochloric acid for 24, 48, and 72 hr at 110°. The hydrolysates were dried by vacuum and dissolved in 0.2 N citrate buffer at pH 2.2. The amino acid compositions of the solutions were determined with a Beckman automatic amino acid analyzer (Model 119 HP).

Results and Discussion

The sodium dodecyl sulfate gel electrophoresis patterns of BDNA-^{14}C-labeled ETP and BHM are shown in Figure 1A and B, respectively. In spite of the complex composition of these systems, there are essentially only two radioactive polypeptide bands, of approximate molecular weights

Table I: Effect of BDNA Labeling on Oxidative Phosphorylation in Bovine Heart Mitochondria.

BDNA at Preincubation (10^{-5} M)	nmoles of Label/mg of Protein		moles of ATP Formed per min per mg of Protein[a]		
	Band I (mol wt 44000)	Band II (mol wt 31000)	Malate + Pyruvate	β-Hydroxybutyrate	Succinate
0 (unwashed)			0.288	0.200	0.068
0 (BSA washed)[b]			0.281	0.078	0.054
1	0.160	0.107	0.139	0.049	0.043
3	0.77	0.53	0.091	0.045	0.039
10	1.38	1.42	0.058	0.050	0.023

[a] For the effect of BDNA labeling on respiration rate, respiratory control ratio, and P:O ratio, see the text. [b] BSA, bovine serum albumin.

44,000 and 31,000, respectively, in the dodecyl sulfate–mercaptoethanol-solubilized samples. The principal radioactive band (band I) in BDNA-^{14}C-labeled bovine heart mitochondria has the same approximate subunit molecular weight (44,000) as the principal radioactive band in DNBP-^{14}C-labeled rat liver mitochondria reported previously (Wang et al., 1973). In the earlier work with DNBP-^{14}C, all the radioactive labels were found by amino acid analysis to be covalently attached to Cys sulfhydryl groups. In the present work with BDNA-^{14}C, radioactive labels were found covalently attached to Cys sulfhydryl as well as to a smaller amount of a hydrolysis product of unknown structure which was adsorbed to the cation-exchanger column even more strongly than arginine.

In order to examine the effect of BDNA labeling on oxidative phosphorylation without the additional complication due to the presence of excess BDNA molecules in the sample, the labeled bovine heart mitochondria were washed with bovine serum albumin solution prior to oxidative phosphorylation measurements as described in a previous report (Copeland et al., 1974). The data summarized in Table I show that labeling of BHM by BDNA suppresses oxidative phosphorylation. With malate + pyruvate as the substrate, we found that bovine serum albumin washing caused negligible damage, but that the efficiency of oxidative phosphorylation decreased by a factor of 5 as the concentration of BDNA at the preincubation stage increased from 0 to 10×10^{-5} M. Raising the concentration of BDNA at the preincubation stage also decreased respiration rate as well as respiratory control ratio (RCR = state 3 rate/state 4 rate), but did not change the P:O ratio. For example, with malate + pyruvate as the substrate and [BDNA] = 0, 5, 10, and 20×10^{-5} M, respectively, we observed the corresponding state 3 respiration rate = 0.159, 0.054, 0.033, and 0.021 μg-atoms of oxygen per min per mg; phosphorylation rate = 0.383, 0.139, 0.088, and 0.044 μmol of ATP per min per mg; RCR = 2.3, 1.23, 1.17, and 1.0 for the BDNA-labeled and bovine serum albumin washed BHM. Thus the P:O ratio remained essentially constant at 2.4 ± 0.2. Similarly the labeling of ETP by BDNA was found to suppress the ATP-driven reduction of NAD^+ by succinate. The results of a typical set of such experiments are summarized in Table II.

The number of nanomoles of covalently bound radioactive label per milligram of mitochondrial protein have been calculated from the known specific radioactivity of the

Table II: Effect of BDNA Labeling on ETP-Catalyzed, ATP-Driven Reduction of NAD^+ by Succinate.

BDNA concentration during preincubation (10^{-5} *M*)	0	1	3	10
nmoles of NADH formed per min per mg of protein	113	95	86	63

Table III: Activities of Respiratory Chain Components in in BDNA-Treated ETP.[a]

	BDNA Concn (10^{-5} *M*)			
Component (s)	0	1	3	10
β-Hydroxybutyrate dehydrogenase	144	113	24.5	0
Malate dehydrogenase[b] (in ETP + supernatant)	2080	2090	2120	2100
NADH-cyt *c* reductase total	560	553	526	532
NADH-cyt *c* reductase (rotenone-insensitive)	23.4	23.7	25.2	23.0
Succinate-cyt *c* reductase	73.1	76.5	79.9	74.1
Cytochrome *c* oxidase	957	922	976	934

[a] The specific activities for the dehydrogenases are expressed in terms of nmoles of hydride ion transferred per min per mg of protein. The enzymatic activities for the reductases and cytochrome oxidase are expressed in terms of nmoles of electrons transported per min per mg of protein. [b] Each sample was prepared by freezing, thawing, and sonicating the chemically modified HBHM for 4 min with 25% duty cycle at 0° and subsequently centrifuging at 38,000*g* for 7 min. The supernatant, which contained ETP and the soluble mitochondrial proteins, was used for each enzyme assay.

BDNA-^{14}C and are also listed in Table I. It may be noticed that after incubation with 1×10^{-5} *M* BDNA, altogether only 0.27 nmol of covalently bound BDNA was found in subunit bands I and II per mg of mitochondrial protein or about 0.4 label/cytochrome aa_3. But the in situ labeling of such a small number of functional groups is sufficient to reduce the rate of phosphorylation of ADP coupled to the mitochondrial oxidation of malate by a factor of 2. The total number of SH groups in BHM, as determined by titrating a sample of dodecyl sulfate treated BHM with 5,5′-dithiobis(2-nitrobenzoic acid), was found to be 89 nmol/mg of protein. Therefore after incubation with 1×10^{-5} *M* BDNA, only 1 out of 330 mitochondrial SH groups was labeled. Thus in situ labeling by BDNA seems to exhibit considerable selectivity at both the protein level and functional group level.

It would be interesting to know whether the observed suppression of oxidative phosphorylation and ATP-driven reduction of NAD^+ by succinate is due to the labeling of substrate dehydrogenases or electron carriers or coupling factors or a combination of these effects. In attempting to find the answer, we studied the effect of BDNA labeling on the biochemical activities of a number of enzymes and electron carriers which are known to be present in BHM as well as in phosphorylating ETP. The results are summarized in Table III.

Inspection of Table III shows that although the activity of β-hydroxybutyrate dehydrogenase diminishes as the concentration of BDNA increases, the activity of malate dehydrogenase is unaffected by BDNA labeling under the experimental conditions. Therefore, we can conclude at least for the case with malate as substrate that the observed suppression of oxidative phosphorylation is not due to labeling of the dehydrogenase. Similarly the activity of succinate–cytochrome *c* reductase is unaffected by BDNA-labeling. Values in Table III also show that the activity of rotenone-sensitive NADH–cytochrome *c* reductase (calculated as the difference between the total and the rotenone-insensitive activities) and the activity of cytochrome *c* oxidase are both unaffected by BDNA labeling. Consequently, we may conclude that neither the observed suppression of phosphorylation coupled to the oxidation of malate nor the observed suppression of ATP-driven reduction of NAD^+ by succinate can be due to the labeling of any of the electron carriers, from NAD^+ to cytochrome oxidase inclusive. Thus the observed decrease in the rate of oxidative phosphorylation appears to be due to the selective labeling of one or more of the coupling factors by BDNA.

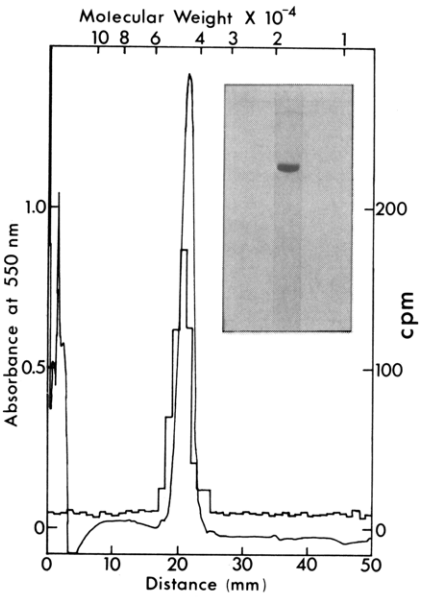


FIGURE 2: Dodecyl sulfate gel electrophoresis pattern of the major radioactive protein isolated from BDNA-^{14}C-labeled bovine heart mitochondria. The smooth curve represents the absorbance of a gel stained with Coomassie Blue and subsequently destained. The step-function trace represents the radioactivity in the same gel determined by controlled slicing and liquid-scintillation counting. The small difference between the maxima of the absorbance and radioactivity curves is within experimental uncertainties. The absorbance changes near the origin are due to reflections at the irregularities of the damaged gel boundary. The photographic insert shows that this boundary is colorless.

In order to explore this last possibility, the BDNA-^{14}C-labeled mitochondrial protein of approximate subunit molecular weight 44,000 (band I in Figure 1B) was isolated by the procedure described in the Materials and Methods section. The pellet obtained from centrifugation of the first crop of *oxidative precipitation* contains radioactive proteins of high purity. Figure 2 shows that dodecyl sulfate gel electrophoresis of this BDNA-labeled protein gave a single polypeptide band of approximate molecular weight 43,000–44,000. The slight difference of the positions of the maxima of the Coomassie Blue dyed protein band and the radioactivity band, respectively, is within the experimental uncertainties of the automatic gel slicer.

The result of amino acid analysis of this labeled protein is summarized in Table IV. Values in Table IV show that except for carboxymethylcysteine (Cm-Cys), the BDNA-labeled protein has within experimental uncertainties the same amino acid composition as the coupling factor F_B (Hi-

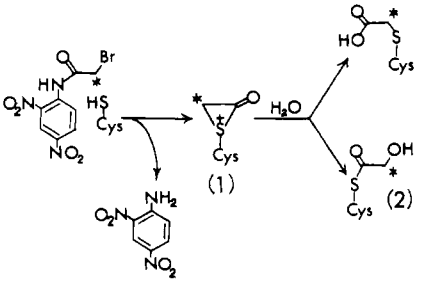
Table IV: Amino Acid Composition of Purified BDNA-Labeled Mitochondial Protein of Subunit Molecular Weight 44,000.

Amino Acid	No. of Residues per Subunit	Amino Acid	No. of Residues per Subunit
S-Carboxymethylcysteine	2.3[b]	Methionine	7
Aspartic acid	40.9	Isoleucine	19.6
Threonine	17.1	Leucine	31.5
Serine	17.8	Tyrosine	11.6
Glutamic acid	39.1	Phenylalanine	13.2
Proline	21.6	Tryptophan[c]	5.3
Glycine	29.3	Lysine	24.4
Alanine	22.1	Histidine	9.0
Valine	24.1	Arginine	30.5

[a] The number of methionine residues per subunit of the BDNA-labeled protein is calculated to be 7, based on the experimental amino acid composition and the approximate subunit molecular weight of 44,000. The number of residues of each of the other amino acids are calculated on the basis of seven methionine residues per subunit. This particular batch of labeled protein was prepared by incubating BHM with 7.5×10^{-5} *M* BDNA and subsequently isolating the labeled protein by the procedure described in the Materials and Methods section. [b] Carboxymethylcysteine was determined by dissolving the BDNA-labeled protein in dodecyl sulfate + 7.5 *M* urea solution containing 1 m*M* mercaptoethanol and incubating with iodoacetic acid (8.5 m*M*) for 2 hr at 40°, followed by dialysis to remove excess iodoacetate and subsequent acid hydrolysis of the protein for amino acid analysis. [c] Tryptophan content was determined with samples with added thioglycolic acid.

gashiyama et al., 1975). The lower value for Cm-Cys in the labeled protein may be due to incomplete carboxymethylation. Except for the minor radioactive product of unknown structure, the only radioactive amino acid derivative found in the acid hydrolysate of the labeled protein is carboxymethylcysteine.

Scheme I



A probable mechanism for the present labeling reaction is summarized in Scheme I in which Cys-SH represents a cysteine residue at the active site and the asterisk indicates the position of ^{14}C atom. While Cm-Cys is a stable radioactive amino acid derivative, compound **2** can be easily hydrolyzed in alkaline solutions to regenerate the nonradioactive Cys. By incubating the BDNA-^{14}C-labeled mitochondria at pH 10 and 22° for 2 hr and subsequently performing dodecyl sulfate gel electrophoresis, it was indeed found that about one-third of the radioactive labels was removed from the mitochondrial proteins.

The close similarity in amino acid composition and the equality of subunit molecular weights suggest that the above radioactive protein was probably produced by in situ labeling of F_B by BDNA. Accordingly, the observed suppression of phosphorylation coupled to the oxidation of malate and of ATP-driven reduction of NAD^+ by succinate may both be attributed to the in situ labeling of F_B by BDNA.

Acknowledgment

We are indebted to Dr. S.-I Tu for suggesting the measurements listed in Table III which are extensions of his unpublished work on DNBP labeling, to Dr. T. Higashiyama for his technical advice, and to Mrs. I. T. Blazy, K. A. Connolly, and B. Stone for their valuable assistance.

References

Copeland, L., Deutsch, C. J., Tu, S.-I., and Wang, J. H. (1974), *Arch. Biochem. Biophys. 160,* 451–457.

Ernster, L., and Lee, C.-P. (1967), *Methods Enzymol. 10,* 729–738.

Gotterer, G. S. (1967), *Biochemistry 6,* 2139–2146.

Hansen, M., and Smith, A. L. (1964), *Biochim. Biophys. Acta 81,* 214–222.

Higashiyama, T., Steinmeier, R. C., Serrianne, B. C., Knoll, S. L., and Wang, J. H. (1975), *Biochemistry,* preceding paper in this issue.

Löw, H., and Vallin, I. (1963), *Biochim. Biophys. Acta 69,* 361–374.

Sottocasa, G. L., Kuylenstierna, B., Ernster, L., and Bergstrand, A. (1967), *J. Cell. Biol. 32* 415–438.

Wang, J. H., Yamauchi, O., Tu, S.-I., Wang, K., Saunders, D. R., Copeland, L., and Copeland, E. (1973), *Arch. Biochem. Biophys. 159,* 785–791.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406–4412.