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Controlled Digestion with Trypsin as a Structural Probe for the N-Terminal Peptide of Soluble and Membranous Cytochrome c_1†

Bernard L. Trumpower* and Aspandiar Katki

ABSTRACT: When purified bovine cytochrome c_1 is digested with trypsin under controlled conditions, the heme polypeptide is preferentially converted from a species of molecular weight 30,600 to a heme polypeptide of molecular weight 29,000. The trypsin sensitive peptide bond is located in the N-terminal region of the cytochrome. Both the reduced and oxidized cytochrome are susceptible to hydrolysis by trypsin at the same locus, but the reduced cytochrome is cleaved at an initial rate approximately twofold greater than the oxidized cytochrome. Membranous cytochrome c_1, as occurring in cytochrome b-c_1 complex or succinate-cytochrome c reductase complex, is not susceptible to trypsin proteolysis under similar conditions, nor after more extensive treatment of the membranes with trypsin, in spite of the fact that cytochrome c_1 presumably comes into contact with cytochrome c at the membrane surface during electron transport. These findings are consistent with a model for the structure of cytochrome c_1 in situ in which the cytochrome is an integral membrane protein, located primarily in the membrane continuum, while still having the heme-containing portion of the protein available at the membrane surface for electron transfer to cytochrome c.

The proteins which make up the electron transport chain appear to fulfill both a structural and a functional role in the inner mitochondrial membrane. Although there have been numerous investigations of the relationship between structure and function of these components of the inner mitochondrial membrane, there has been no precedent attempt to elucidate the three-dimensional structure of a single mitochondrial membrane protein in situ.

Cytochrome c_1 is particularly suitable as a subject for investigation of protein structure within the mitochondrial membrane. In addition to its obligatory function in electron transport, cytochrome c_1 is a membrane protein of a type which cannot be dissociated from the inner membrane without concomitant destruction of the membrane structure and loss of associated membrane activities. It thus differs from such mitochondrial proteins as cytochrome c and the AT-

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Pase, which can be reversibly dissociated from the inner membrane while retaining a membrane with the latent capability to catalyze electron transport and oxidative phosphorylation (Jacobs and Sanadi, 1960; Racker, 1970).

Although cytochrome c_1 is not released from the inner membrane by high ionic strength, nor by ultrasonic irradiation, it is extracted by anionic detergent (Yu et al., 1972) or by chaotropic solutes (B. Trumpower, unpublished experiments) which disrupt hydrophobic bonds (Hatefi and Hanstein, 1974). Thus c_1 appears to be located in the membrane continuum in a manner such that it contributes to the structure of the membrane, but it remains to be established whether c_1 is an *integral* or *peripheral* membrane protein as defined by Singer (1971).

Available evidence indicates that there is direct electron transfer from cytochrome c_1 to cytochrome *c*, which implies some form of specific physical contact between membranous c_1 and membrane-bound cytochrome *c*. The formation of a complex between purified cytochromes c_1 and *c* supports the notion of such specific protein–protein interaction (Kaminsky et al., 1974).

Although the exact mechanism of electron transfer within cytochrome *c* is still incompletely understood, the availability of extensive structural information (Dickerson, 1972) and recent findings with site-specific antibodies (Smith et al., 1973) have made it possible to postulate specific loci on cytochrome *c* as sites of interaction with cytochrome c_1. By contrast, very little information is available regarding the structure of cytochrome c_1 in the mitochondrial membrane, although the interaction of these two proteins is of particular interest as an example of protein–protein interaction at a membrane surface. For instance, although it is generally thought that cytochrome c_1 interacts with cytochrome *c* at the exterior surface (C side) of the inner mitochondrial membrane (Racker, 1970), there is also evidence that c_1 will react with ferricyanide only when the inner surface (M side) of the membrane is accessible to the reagent, which does not penetrate the membrane (Harmon et al., 1974). In addition, if cytochrome c_1 accepts electrons from a redox component within the membrane continuum, such as cytochrome *b*, the heme peptide region of c_1 may undergo considerable movement within the membrane during electron transfer. The nature of intramembrane movement of cytochromes during electron transport has been speculated upon (Chance et al., 1968) but remains unsupported by definitive experimental evidence. Furthermore, if electron transfer between membranous c_1 and bound c proceeds through peripheral contact of the porphyrin rings (Salemme et al., 1973), the heme peptide region of cytochrome c_1 must be accessible to cytochrome *c* at the membrane surface.

We have undertaken experiments to elucidate selected aspects of the structure of cytochrome c_1 as it exists in the inner mitochondrial membrane. This report describes results of studies in which we have developed controlled digestion with trypsin as a structural probe with purified and membranous cytochrome c_1.

Experimental Section

Preparation of Resolved Membrane Complexes. Complex III was prepared from bovine heart mitochondria by Hatefi's procedure (Hatefi et al., 1962) as modified by Ragan and Racker (1973). The complex III contained 4.1 nmol of cytochrome c_1 and 7.0 nmol of cytochrome *b* per mg of protein. After reconstitution with succinate dehydrogenase and phospholipid (Yamashita and Racker, 1969) the preparation exhibited succinate–cytochrome *c* reductase activity of 6–8 μmol per min per mg.

Succinate–cytochrome *c* reductase complex was prepared as previously described (Trumpower and Katki, 1975). The reductase complex contained 1.8 nmol of cytochrome c_1 and 3.4 nmol of cytochrome *b* per mg of protein and exhibited a succinate–cytochrome *c* reductase activity of 2.8 μmol per min per mg. The cytochrome *c* reductase activity of the reductase complex was not dependent upon reconstitution with succinate dehydrogenase and phospholipid. The succinate–cytochrome *c* reductase activity of both the reconstituted complex III and the reductase complex was completely inhibited by antimycin.

Purification of Cytochrome c_1. Succinate–cytochrome *c* reductase complex was depleted of succinate dehydrogenase according to King (1967), suspended at 15 mg/ml in 0.1 *M* sodium phosphate–0.5 m*M* EDTA (pH 7.4), and reduced by addition of 2 mg/ml of sodium dithionite. All subsequent manipulations were performed at 4°. The suspension was adjusted to pH 8.0 with concentrated NH_4OH, after which 10% sodium cholate and 5 *M* guanidine·HCl were added, in sequence, to obtain a mixture containing 9.45 mg/ml of protein, 0.7% cholate, and 1.5 *M* guanidine. After stirring 30 min, the mixture was diluted with 0.5 volume of phosphate–EDTA buffer and centrifuged 30 min at 78,500*g*. The orange supernate was decanted and dialyzed 4 hr against 20 volumes of 20 m*M* sodium phosphate–0.5 m*M* dithiothreitol–0.1 m*M* EDTA (pH 7.4). The dialysate was centrifuged 30 min at 78,500*g*, after which the yellow supernate was discarded and the membranous c_1 was suspended to 5 mg/ml in 0.1 *M* sodium phosphate–0.5 m*M* EDTA (pH 7.4).

The suspension of membranous c_1 was adjusted to pH 8.6 with NH_4OH, after which 20% sodium cholate and 5 *M* guanidine were added, in sequence, to obtain a mixture containing 1.1% cholate and 1.14 *M* guanidine. After the mixture was stirred 60 min, the solubilized c_1 was recovered by centrifugation for 20 min at 44,000*g*.

Solid ammonium sulfate was added to the c_1-containing extract to obtain 42% saturation (242 g/l.) and the resulting precipitate was separated by centrifugation and discarded. Additional ammonium sulfate was added to the supernate to obtain 70% saturation (180 g/l.) and the c_1 thus precipitated was collected by centrifugation and redissolved at 2 mg/ml in 0.1 *M* sodium phosphate–0.5% cholate–0.5 m*M* EDTA (pH 7.4). The partially purified c_1 had an absorbance ratio A_{417}/A_{278} = 1.2, a heme content of 15 nmol/mg, and was recovered in 65–70% yield from the reductase complex.

The c_1 was precipitated from phosphate–cholate by addition of an equal volume of saturated, neutralized ammonium sulfate (3.9 *M*) and collected by centrifugation. The c_1 was dissolved at 2 mg/ml in 0.1 *M* sodium phosphate–0.5% cholate–1% β-mercaptoethanol–0.5 m*M* EDTA (pH 7.4). Saturated ammonium sulfate was added to obtain 33% saturation and the resulting precipitate removed by centrifugation. Cytochrome c_1 was precipitated by further addition of saturated ammonium sulfate to obtain 50% saturation. The fractionation with 0–33 and 33–50% ammonium sulfate was repeated two times, after which the c_1 was dissolved in 0.1 *M* sodium phosphate–0.5 m*M* EDTA (pH 7.4), precipitated with 50% ammonium sulfate to remove excess cholate, and dissolved at 30–40 nmol/ml in 20 m*M* potassium phosphate (pH 7.0). At this stage of purification

the c_1 had an absorbance ratio A_{417}/A_{278} = 1.50 or greater, a heme content of 18 nmol/mg, and was recovered in 50% yield.

A suspension of calcium phosphate gel (Singer and Kearney, 1950) was added to obtain 5 mg of gel/nmol of c_1. The gel, with adsorbed c_1, was recovered by centrifugation 5 min at 12,000*g*. The supernate was discarded and the gel was washed two times with 100 m*M* potassium phosphate (pH 7.0), after which c_1 was eluted with a minimum volume of 0.5 *M* potassium phosphate (pH 8.0). The pure c_1 was precipitated by addition of saturated ammonium sulfate to obtain 65% saturation and dissolved in 0.1 *M* sodium phosphate–0.5 m*M* EDTA (pH 7.4) for storage at −70°. The recovery of pure c_1, having an absorbance ratio A_{417}/A_{278} = 2.50, was 13–16% of that in the reductase complex. On repeated freezing and thawing the ratio A_{417}/A_{278} dropped to 2.20–2.30, but the small amount of denatured c_1 causing this decrease could be removed by readsorption to calcium phosphate gel from 20 m*M* phosphate (pH 7.0) and direct elution with 500 m*M* phosphate (pH 8.0). The c_1 thus obtained appears to be identical in purity with that of Yu and coworkers (1972). Selected additional characteristics of pure c_1 are reported below.

Treatment of Cytochrome c_1 and Membrane Complexes with Trypsin. Controlled digestions with trypsin were routinely performed by incubating 2 mg/ml of cytochrome c_1 or 2 mg/ml of complex III with various amounts of trypsin for 30 min at 37°. Exceptions to this procedure and the amounts of trypsin used in incubations are specified in the figure legends.

To measure initial rates of digestion of ferro- and ferricytochrome c_1 by trypsin, the ferricytochrome was obtained by preincubating 3.75 nmol of c_1 with 5×10^{-2} nmol of cytochrome *c* and 5×10^{-2} nmol of cytochrome oxidase in 100 μl of 100 m*M* NaP_i–0.5 m*M* EDTA (pH 7.4) for 20 min prior to addition of trypsin. Ferrocytochrome c_1 was obtained by preincubating 3.75 nmol of c_1 with 4×10^{-2} nmol of succinate–cytochrome *c* reductase complex and 1 μmol of succinate in 100 m*M* NaP_i–0.5 m*M* EDTA (pH 7.4) for 20 min prior to addition of trypsin. Initial rates of hydrolysis by trypsin were measured at 25°; the reactions were initiated by addition of 2 μg of trypsin/mg of c_1 and stopped at carefully measured time intervals by addition of 10 mg of soybean trypsin inhibitor/mg of trypsin. The amount of heme protein remaining after trypsin treatment was calculated from profiles of stained electrophoresis gels in which the resolution of the 30,600 and 29,000 polypeptides was increased by conducting the electrophoresis for 8 hr at 6 mA per gel. The amount of sample applied to each gel was equivalent to 1.5 μg of c_1 prior to trypsin treatment.

Analytical Methods. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to Weber and Osborn (1969) with the exception that the proteins were denatured at 1 mg/ml in 2% β-mercaptoethanol and 5% sodium dodecyl sulfate as discussed below. Bovine serum albumin, liver glutamate dehydrogenase, ovalbumin, yeast alcohol dehydrogenase, carbonic anhydrase, chymotrypsinogen, myoglobin, ribonuclease A, and cytochrome *c* were employed as molecular weight standards (Weber and Osborn, 1969).

Free electrophoretic mobilities and retardation coefficients were measured in the buffer system of Weber and Osborn (1969), using acrylamide gels formulated with C = 3.5 and T = 5–10 as described by Banker and Cotman (1972). Ferguson-type plots were calculated for glutamate dehydrogenase, ovalbumin, yeast alcohol dehydrogenase, carbonic anhydrase, and chymotrypsinogen, in addition to cytochrome c_1.

Amino acid analysis was performed essentially as described by Spackman and coworkers (1958) on a Beckman 120C amino acid analyzer with modifications for single column operation. Heme-containing polypeptides were isolated from c_1 before and after trypsin treatment by preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis and excess detergent was removed by dialysis. The samples were hydrolyzed in constant boiling HCl containing 1% phenol (Sanger and Thompson, 1963) in evacuated, sealed tubes at 106°. Calculations were performed according to Coulson (1972).

N-Terminal amino acid analysis was performed with dansyl chloride on performic acid oxidized samples as recommended by Gray (1972). Dansyl derivatives were identified by thin-layer chromatography on polyamide sheets, using three solvent systems and dansylated amino acid standards (Gray, 1972).

Heme-containing polypeptides were identified after sodium dodecyl sulfate polyacrylamide gel electrophoresis by modification of a staining procedure originally used to detect hemoglobin in starch gel electrophoresis (Haut et al., 1962). After sodium dodecyl sulfate polyacrylamide gel electrophoresis the acrylamide gels were fixed for 24 hr at 37° in 50% methanol–10% acetic acid. The fixed gels were transferred to a benzidine solution, prepared immediately prior to use by mixing 1 g of benzidine·HCl, 30 ml of water, 20 ml of glacial acetic acid, and 50 ml of 95% ethanol. Each gel was soaked 3 hr at 25° in 40 ml of benzidine solution, after which excess benzidine was decanted and the gels were transferred to 3% hydrogen peroxide for color development. Heme-containing polypeptides immediately appeared as dark blue bands, visualized against a colorless background. Development of the blue color was transient and changed to lavender after 1 hr at 25°. The heme-containing polypeptide from 5 μg of c_1 could be detected, although larger amounts were stained for photographic record. Protein standards which did not contain heme did not react with the benzidine–peroxide stain.

Absorption spectra were obtained at ambient temperature on a Cary 118C spectrophotometer. Cytochrome *b* and c_1 content of resolved membrane complexes was measured by difference spectroscopy (Berden and Slater, 1970). Heme content and concentrations of soluble cytochrome c_1 were calculated from the 417-nm absorption maximum of the ferrocytochrome, using E_{417} = 143 mM^{-1} cm^{-1} (Yu et al., 1972).

Total phosphate was measured after complete acid hydrolysis (Kagawa and Racker, 1966) and protein was determined according to Lowry et al. (1951).

Reagents. Bovine pancreatic trypsin (2× crystallized), soybean trypsin inhibitor, *N*-ethylmorpholine, dansyl chloride, amino acid standards, and proteins used as molecular weight standards were obtained from Sigma Chemical Co. Polyamide layer sheets, manufactured by Cheng Chin Trading Co., Ltd. (Taiwan), were obtained from Gallard-Schlesinger Chemical Corp., New York. Reagents for electrophoresis, including Coomassie Brilliant Blue, were obtained from Bio-Rad and used without further purification.

Results

Selected Properties of Purified Cytochrome c_1. Cytochrome c_1 as purified by us exhibited an absorbance ratio

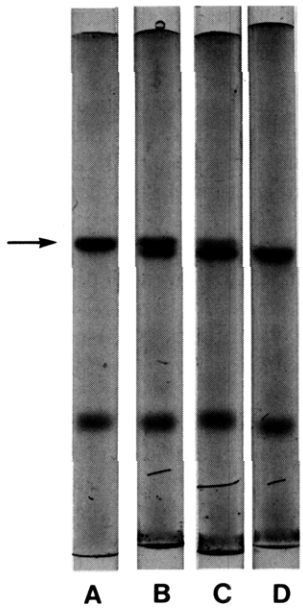

FIGURE 1: Electrophoresis of cytochrome c_1 before and after controlled digestion with trypsin. Sample A is a "zero time" control in which c_1 was incubated 30 min at 37° in the absence of trypsin, after which 2.0 μg of trypsin inhibitor and 1.0 μg of trypsin per mg of c_1 were sequentially added. In samples B, C, and D the c_1 was incubated 30 min at 37° with 0.1, 0.2, and 1.0 μg of trypsin/mg of c, respectively. The arrow indicates the migration position of the 30,600 heme-containing subunit.

A_{417}/A_{278} = 2.50 and contained 25 nmol of heme/mg of protein; both of these purity indexes are identical with those reported by Yu et al. (1972). In addition, we have observed that the absorbance ratio A_{417}/A_{370} is a useful criterion of purity. This ratio relates the 417-nm Soret absorption maximum of the ferrocytochrome to a trough which occurs at a wavelength at which non-heme iron proteins show appreciable absorbance (cf. Palmer and Brintzinger, 1972). Pure ferrocytochrome c_1 has an absorbance ratio A_{417}/A_{370} = 12.0. The c_1 was analyzed for total phosphate content and no phosphate could be detected, using amounts of protein which would have permitted detection of 1 mol of phosphate/30 mol of heme. The purified c_1 thus appears to be free of phospholipid, in contrast to the preparation of Bomstein and coworkers (1961). Comparable data are not available for the preparation of Yu et al. (1972).

Yu and coworkers (1972) reported a molecular weight of 29,000 for the heme-containing polypeptide of c_1 but have not shown the appearance of the purified cytochrome after dodecyl sulfate polyacrylamide gel electrophoresis analysis as used in the molecular weight measurements. We observed that under conditions routinely employed for dodecyl sulfate polyacrylamide gel electrophoresis analysis the c_1 behaved anomalously, in that the heme-containing polypeptide occasionally migrated as two species with apparent molecular weights of 27,000 and 32,000. The ratio of these two species varied widely; in some instances only one heme polypeptide was observed, in which case its molecular weight varied from 27,000 to 32,000 and as much as 20–40 μg of pure c_1 had to be applied to the electrophoresis gel for adequate detection by Coomassie Brilliant Blue. These considerations and our observations on the sensitivity of c_1 to proteolysis prompted us to assess more extensively the molecular weight of the heme polypeptide of cytochrome c_1.

The anomalous behavior of c_1 on dodecyl sulfate polyacrylamide gel electrophoresis was not eliminated by inclusion of 8 *M* urea during denaturation, by sulfonation (Chan, 1968), nor by extraction with acetone prior to exposure to sodium dodecyl sulfate. Further experimentation revealed that after denaturation in the presence of 2% β-mercaptoethanol and 5% dodecyl sulfate the heme-containing polypeptide migrated as a sharply defined species of constant molecular weight on dodecyl sulfate polyacrylamide gel electrophoresis, and documentation of the purity of the c_1 became possible as shown in Figure 1. The molecular weight of the heme-containing subunit, whose identity is established below, was determined to be 30,600 (±500), and that of the non-heme-containing subunit to be 14,000 (±400).[1] From densitometer tracings of the stained gels, we calculated a ratio of 2.5:1 for the two subunits (cf. Yu et al., 1972) but since the staining of different polypeptides by Coomassie Brilliant Blue may not be equivalent, this ratio is not reliable as a basis for calculating subunit stoichiometry.

Effect of Trypsin on Soluble Cytochrome c_1. When purified, soluble cytochrome c_1 is submitted to controlled digestion with trypsin, the protease preferentially hydrolyzes a single peptide bond in the heme-containing subunit. Figure 1 shows the results of an experiment in which c_1 was incubated with varying amounts of trypsin for a constant period of time, after which the reaction was stopped by addition of trypsin inhibitor. There occurs a progressive conversion of the 30,600 polypeptide to a first generation product of molecular weight 29,000. The small peptide thus liberated, accounting for a molecular weight increment of approximately 1600, would not be expected to be retained in the electrophoresis gel and is thus not directly demonstrable.

During a 30-min incubation, approximately 50% of the heme subunit is converted to the first tryptic product by 0.1 μg of trypsin/mg of cytochrome, and complete conversion is obtained with 1 μg of trypsin. The small amounts of trypsin required and the efficiency of trypsin inhibitor essentially preclude the possibility that this proteolysis might be due to an unrecognized chymotrypsin contaminant.

The electrophoresis profile shown in Figure 1 includes a "zero time" sample in which c_1 was incubated with trypsin inhibitor prior to addition of trypsin. This control excludes the possibility that the dodecyl sulfate added as denaturant prior to electrophoresis might induce the observed susceptibility to protease (cf. Wallach, 1972).

Covalently bound heme is associated with the 30,600 polypeptide of the native cytochrome and is retained in the 29,000 polypeptide resulting from trypsin treatment. The heme could be detected by scanning unstained electrophoresis gels at 415 nm or by reaction with a heme-specific stain as shown in Figure 2. No heme is associated with the subunit of molecular weight 14,000.

Amino acid analysis and N-terminal analysis of the

[1] These molecular weights were determined by the sodium dodecyl sulfate polyacrylamide gel electrophoresis procedure, incorporating the conditions of denaturation described above. We tested, and confirmed, the validity of the sodium dodecyl sulfate polyacrylamide gel electrophoresis method for determining the molecular weight of cytochrome c_1 by measuring free electrophoretic mobilities and retardation coefficients of the cytochrome and protein standards in the presence of dodecyl sulfate as recommended by Banker and Cotman (1972). The usefulness of such analysis in validating the sodium dodecyl sulfate polyacrylamide gel electrophoresis procedure for membrane proteins is well illustrated with the subunits of cytochrome oxidase (Poyton and Schatz, 1975). Carbonic anhydrase, molecular weight 29,000, was included as one of the standard proteins. We thus conclude that the value of 29,000 reported for the molecular weight of the heme-containing subunit of c_1 (Yu et al., 1972) is somewhat too low.

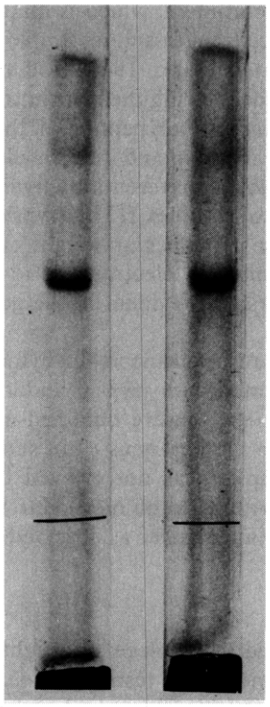

FIGURE 2: Detection of the heme-containing subunit of cytochrome c_1 after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. A 50-μg sample of c_1, not treated with trypsin, was applied to the gel on the left; an identical quantity of c_1 was applied to the gel on the right after incubation with 1.0 μg of trypsin/mg of c_1. The wire marks the migration position of the tracking dye.

heme-containing polypeptides before and after trypsin treatment are summarized in Table I. Prior to trypsin treatment no free N-terminal amino acid could be detected in the heme subunit, suggesting that cytochrome c_1 has a blocked N-terminus, as does bovine cytochrome c (Eck and Dayhoff, 1966). After controlled digestion with trypsin the 29,000 polypeptide exhibited an N-terminal lysine, indicating that trypsin hydrolyzes a lysyllysyl or arginyllysyl peptide bound in the N-terminal region of the heme protein. The appearance of only a single N-terminal residue indicates that there is very little hydrolysis, if any, at the carbonyl group of the lysine which appears as the N-terminus in the tryptic product.

The amino acid content of the heme subunit of the native cytochrome is of interest because of several apparently unique properties which may relate to the structure of c_1 in the membrane and its role in electron transport. There is a relatively high content of proline, and glycine plus proline account for 17% of the residues of the heme subunit. The high content of glutamate plus aspartate suggests that c_1 is an acidic protein, although an undetermined number of the carboxyl groups may exist as amides. The purified c_1 does exhibit properties expected of an acidic protein. Cytochrome c_1 quantitatively absorbs to DEAE-cellulose at pH 8.0 and remains bound to the ion exchange resin throughout a linear gradient until 0.75 M NaCl is obtained (B. Trumpower and A. Katki, unpublished observations). Also, the purified cytochrome is reversibly precipitated from solution at pH 5.6 and can be subsequently redissolved in buffer at neutral pH, indicating that the insolubility is not associated with denaturation.

It is of particular interest that the heme subunit of c_1 contains four half-cystine residues. Two cysteines are involved in thiol ether linkages to the porphyrin ring of the heme (Yu et al., 1972) as in all c-type cytochromes, but the occurrence of two cysteine residues outside of the thioether linkages is unusual.

Table I: Amino Acid Content of Cytochrome c_1 Heme Polypeptide before and after Controlled Digestion with Trypsin.[a]

Residue	Before Trypsin	After Trypsin
Aspartate	23.2	23.0
Threonine	9.2	8.4
Serine	15.8	14.2
Glutamate	27.9	25.6
Proline	24.8	22.4
Glycine	19.4	19.1
Alanine	21.9	21.7
Valine	16.6	16.5
Methionine	8.5	8.3
Isoleucine	5.0	5.2
Leucine	28.3	27.3
Tyrosine	15.0	13.6
Phenylalanine	9.9	9.5
Histidine	9.6	8.2
Lysine	12.9	11.7
Arginine	16.7	15.1
Tryptophan[b]		
Cysteic acid[c]	4.3	4.0
N-terminal	None detected	Lysine

[a] Data are expressed as residues per molecule of heme-containing polypeptide based on molecular weights determined by sodium dodeyl sulfate polyacrylamide gel electrophoresis analysis and corrected for the contribution of heme to the measured molecular weight. [b] Not determined. [c] Cysteic acid was determined after performic acid oxidation and normalized against aspartic acid.

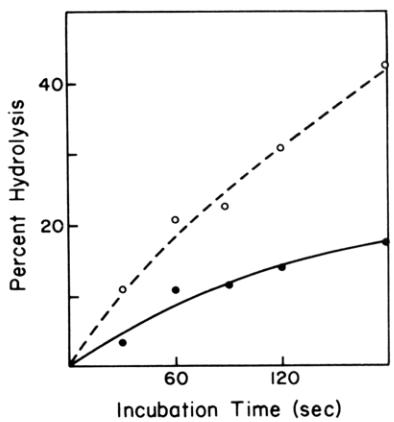

FIGURE 3: Effect of the redox status of the heme on the rate of proteolysis of the heme-containing subunit of cytochrome c_1. The extent of hydrolysis was calculated from the disappearance of heme-containing subunit after exposure of ferro- or ferricytochrome c_1 to 2.0 μg of trypsin/mg of c_1 for various incubation times as indicated. The solid line depicts the hydrolysis of the ferricytochrome c_1.

The increment in amino acid content of the heme-containing polypeptide due to trypsin treatment is too small to allow any conclusion to be drawn regarding the nature of the N-terminal peptide which is liberated.

Although amino acid content is of limited value in distinguishing hydrophobic vs. hydrophilic proteins, the "polarity" of the heme subunit, as defined by Capaldi and Vanderkooi (1972), is 42.9%, which is comparable to one of the two hydrophobic subunits of cytochrome oxidase (Poyton and Schatz, 1975).

The initial rate of hydrolysis of the N-terminal peptide is affected by the oxidation state of the heme protein. As shown in Figure 3, the ferrocytochrome c_1 is cleaved by

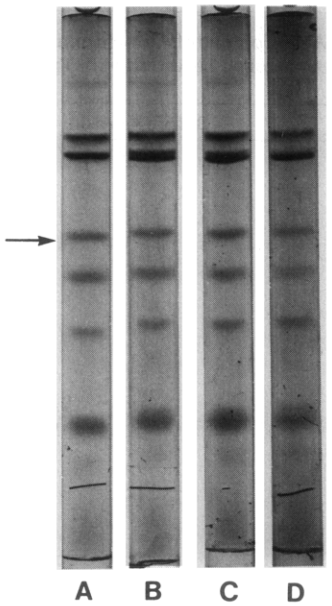

FIGURE 4: Electrophoresis of cytochrome b–c_1 complex before and after controlled digestion with trypsin. Sample A is a "zero time" control in which the complex was incubated 30 min at 37° in the absence of trypsin, after which 2.0 μg of trypsin inhibitor and 1.0 μg of trypsin per mg of complex were sequentially added. In samples B, C, and D the complex was incubated 30 min at 37° with 0.1, 0.2, and 1.0 μg of trypsin/mg of complex. The arrow indicates the 30,600 heme-containing subunit of cytochrome c_1.

trypsin at an initial rate approximately twofold greater than the ferricytochrome. Although the redox status of the heme affects the lability of the N-terminal peptide to trypsin, trypsin cleavage of the N-terminal peptide causes no detectable change in accessibility of the heme to external ligands. The trypsin-modified c_1 was identical with the native cytochrome in that it showed no reactivity with KCN, CO, or O_2.

Effect of Trypsin on Cytochrome c_1 in Resolved Membrane Fragments. Resolved segments of the inner mitochondrial membrane, such as succinate–cytochrome c reductase complex or cytochrome b–c_1 complex (complex III), offer several advantages in attempts to examine cytochrome c_1 in its native membrane environment. These lipoprotein complexes retain relevant biological activities, such as enzymatic reduction of cytochrome c (Hatefi, 1966), and are sufficiently resolved to permit recognition of c_1 amidst a small number of functionally related proteins. As shown in Figure 4 complex III contains six polypeptides, having molecular weights 48,200, 44,000, 30,600, 25,000, 21,000, and 14,000. The heme-containing subunit of c_1 is clearly distinguishable and well resolved from other polypeptides.

The heme subunit of cytochrome c_1 in the membranous complex is not accessible to trypsin when the complex is exposed to trypsin under conditions which lead to proteolysis of the soluble c_1 as shown in Figure 4. The amount of heme subunit in the complex treated with trypsin remains constant and the first generation tryptic product expected upon trypsin cleavage of the soluble c_1 is not observed. In the experiment shown, for purposes of comparison, the complex was treated with trypsin under identical conditions as employed with the soluble c_1; however, we have tested as much as 25 μg of trypsin/mg of membrane complex and detected no proteolysis of the endogenous c_1. The inaccessibility of membranous c_1 to trypsin could not be attributed to a masking effect of cytochrome c since the resolved complex contains no cytochrome c, which is quantitatively removed by the high ionic strength solutes employed in the complex preparation (Hatefi et al., 1962) and no exogenous cytochrome c was added during the trypsin treatment.[2]

The most abundant polypeptide of the cytochrome b–c_1 complex, whose identity and role in electron transport is currently unknown, is preferentially hydrolyzed during controlled digestion of complex III by trypsin. This polypeptide is converted from a molecular weight of 44,000 to 43,500. The small increment in electrophoretic mobility accompanying this conversion is evident in the gels shown in Figure 4.

When the less resolved succinate–cytochrome c reductase complex was treated with trypsin under these same conditions, the same results were obtained as shown here with complex III. The membranous c_1 in succinate–cytochrome c reductase complex was not cleaved by trypsin, while a predominant polypeptide of molecular weight 44,000 was digested in the same manner as observed with complex III.

Discussion

We reevaluated the molecular weight of the heme subunit of cytochrome c_1 and tested the reliability of the dodecyl sulfate polyacrylamide gel electrophoresis procedure with c_1 for several reasons: (1) the heme subunit is highly sensitive to protease and is thus hydrolyzed to a polypeptide whose molecular weight we find to be identical with that previously reported for the heme-containing subunit (Yu et al., 1972); (2) while the appearance of c_1 on dodecyl sulfate polyacrylamide gel electrophoresis has not been previously documented, we observed that pure c_1 behaved in a manner uniquely different from protein standards during dodecyl sulfate polyacrylamide gel electrophoresis measurements; and (3) the dodecyl sulfate polyacrylamide gel electrophoresis procedure has previously been noted to yield erroneous molecular weights for certain membrane proteins (Banker and Cotman, 1972). After denaturing the cytochrome with fivefold greater concentration of dodecyl sulfate than is routinely employed, this membrane protein conformed to expected behavior on dodecyl sulfate polyacrylamide gel electrophoresis analysis. We were thus able to document the purity of cytochrome c_1 and establish a reliable molecular weight for the heme-containing subunit. Recent evidence indicates that cationic sites on the polypeptide are of primary importance in dodecyl sulfate binding to proteins (Igou et al., 1974), and thus the high concentrations of dodecyl sulfate required with c_1 may reflect inefficient binding of dodecyl sulfate to this acidic protein.

Although the molecular weight increment from 30,600 to 29,000 is too small to allow a comparison of values from different laboratories, our findings do suggest that caution is warranted to protect c_1 from protease digestion during its purification. This potential problem is exasperated by the fact that the native and tryptic c_1 show apparently identical spectral properties and minute differences in molecular weights of the heme subunit.

Controlled digestion with trypsin, in combination with dodecyl sulfate polyacrylamide gel electrophoresis, provides

[2] The absence of cytochrome c in preparations of complex III and succinate–cytochrome c reductase complex is also evident from the undistorted absorption maximum at 553.2 nm of reduced c_1 in difference spectra of these complexes and the inability of cytochrome c oxidase to oxidize the membranous c_1 in the absence of added cytochrome c.

a specific and sensitive probe for selected aspects of the structure of cytochrome c_1. The usefulness of this approach stems from the finding that the isolated protein contains a susceptible peptide bond in the N-terminal region of the heme subunit which is preferentially hydrolyzed to completion prior to any subsequent proteolysis.

The results indicate that a lysyllysyl or arginyllysyl bond in the N-terminal region of the heme subunit is accessible at the surface of the isolated protein and during electron transfer a conformational change occurs which results in an increased exposure of this peptide in the ferrocytochrome. It will be of particular interest to test our interpretation of this result with the aid of the complete amino acid sequence and crystallographic structure of bovine c_1 as they become known. Cytochrome *c* also exhibits a susceptibility to protease which varies with the redox status of the heme, but in the opposite manner than observed with c_1. Ferricytochrome *c* is more labile to bacterial protease and to trypsin than is ferrocytochrome *c* (Yamanaka et al., 1959).

Unfortunately, due to the limited quantity of available material, very little information is available regarding the N-terminal peptide which is released from the heme polypeptide by trypsin. It can only be inferred that this peptide has a blocked N-terminal residue, a lysine or arginine C-terminal residue, does not contain heme or cysteine, and consists of approximately 15 amino acids.

It will be of interest to determine the location of the c_1 heme moiety with respect to the site at which trypsin hydrolysis occurs. In bovine cytochrome *c* the heme is attached to the polypeptide through thioether linkages to cysteines at positions number 14 and number 17. It may be that the heme linkage in bovine c_1 is further removed from the N-terminus than in cytochrome *c*.

The distinguishing feature of the amino acid content of the heme subunit of c_1 is the high content of proline, the large number of acidic residues, and two cysteine residues in addition to those in thioether linkages to the heme. The content of acidic residues is consistent with the observed interaction of soluble c_1 and cytochrome *c*, which form a complex which is ionic in character (Kaminsky et al., 1974). It seems likely that c_1 is arranged in the inner mitochondrial membrane in an orientation which exposes acidic residues to the membrane surface for ionic binding of cytochrome *c*. Our finding that c_1 in resolved membrane complexes is not hydrolyzed by trypsin, but is reactive in electron transfer to cytochrome *c*, is fully consistent with this view.

The occurrence of two cysteine residues in the heme subunit of c_1 is without precedent among mammalian *c*-type cytochromes. By chemical derivatization of the purified cytochrome it has been established that these do not exist as a disulfide (B. Trumpower, unpublished experiments). However, it may be that the cysteines of c_1 in the inner membrane form an intra- or interprotein disulfide; such an occurrence would explain the necessity of disulfide reducing agents in the solubilization of c_1 (Yu et al., 1972).

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Replacement of Acyl and Alk-1-enyl Groups in *Clostridium butyricum* Phospholipids by Exogenous Fatty Acids†

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ABSTRACT: The effect of exogenous unsaturated fatty acids on the acyl and alk-1-enyl group composition of the phospholipids of *Clostridium butyricum* has been examined. Unsaturated fatty acids support the growth of this organism in the absence of biotin. When cells were grown at 37° in media containing oleate or linoleate and a Casamino acid mixture containing traces of biotin, the exogenous fatty acids were found mainly in the alk-1-enyl chains of the plasmalogens with less pronounced incorporation into the acyl chains. However, at 25° in this medium, both the acyl and alk-1-enyl chains contained substantial amounts of the 18:1 supplement plus the C_{19}-cyclopropane chains derived from it. Alk-1-enyl chains in all the major phosphatide classes showed a uniformly high substitution by the oleate supplement in cells grown at 37°. The oleate and C_{19}-cyclopropane content of the acyl chains was more variable among the phosphatide classes. At 37°, *trans*-9-octadecenoic acid (elaidic acid) also supported growth and was incorporated into both acyl and alk-1-enyl chains at a high level. When cells were grown on oleate at 37° in media containing biotin-free Casamino acids, both the acyl and alk-1-enyl chains had a high level of 18:1 plus C_{19}-cyclopropane chains. In the cells grown at 37° with oleate substantial changes were seen in the phospholipid class composition. There was a large decrease in the ethanolamine plus *N*-methylethanolamine plasmalogens with a corresponding increase in the glycerol acetals of these plasmalogens. The glycerol phosphoglycerides were also significantly lower with the appearance of an unknown, relatively nonpolar phospholipid fraction.

There is now substantial evidence that phospholipids play a major role in the structure and function of biological membranes (Cronan and Vagelos, 1972; Singer and Nicolson 1972). The influence of the physical state of membrane lipids on membrane assembly and membrane function has been studied extensively in microbial unsaturated fatty acid auxotrophs in which the fatty acid composition of the membrane lipids can be manipulated by changing the fatty acid supplement in the growth medium (Machtiger and Fox, 1973). It is generally assumed that thermal phase transitions in biological membranes largely reflect the transitions of the fatty acyl chains of the membrane phospholipids, suggesting that the physical properties of the hydrocarbon chains are one of the principal factors involved in regulating membrane fluidity (Esfahani et al., 1969; Overath et al., 1970; Gitler, 1973). Several laboratories have correlated the temperature dependence of solute transport in bacteria with the physical state of the membrane lipids in cells possessing altered acyl group composition (Machtiger and Fox, 1973; Linden et al., 1973; Overath and Traüble, 1973).

Clostridium butyricum is an obligate anaerobe in which plasmalogens (derivatives of 1-alk-1′-enyl-*sn*-glycerol 3-phosphate) constitute more than half of the total phospholipids. We are seeking to determine if the plasmalogens of these anaerobic bacteria contribute to the fluid properties of the cell membrane, and, if so, what effect they have on the functions of the cell membrane. As first steps toward the alteration of the lipid composition in *C. butyricum,* we have tried to change the apolar chain composition of the membrane lipids by two approaches: (1) by growing the cells at various temperatures, and (2) by supplementing the growth medium with unsaturated fatty acids in place of biotin. The results obtained upon changing the growth temperature suggest that plasmalogens do participate in an adaptive response of the cell membrane lipids (Khuller and Goldfine, 1974). In this paper, the results of the second approach are reported, which show that both the acyl and the alk-1-enyl[1] group composition in *C. butyricum* polar lipids can be manipulated by supplementing the growth medium with unsaturated fatty acids in the absence of biotin or in the presence of suboptimal levels of biotin.

Experimental Section

Materials. *cis*-9-Octadecenoic, *trans*-9-octadecenoic, and linoleic acids were obtained from Nu-chek Prep, Elysian, Minn. Pentadecanoic acid and NIH-D standard fatty

† From the Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19174. *Received April 22, 1975.* This investigation was supported by a grant from the National Institute of Allergy and Infectious Diseases (AI-08903). A preliminary report of this work was presented at the 12th International Congress for Fat Research, Milan, Italy, September, 1974 (Goldfine et al., 1975).

[1] In this paper alk-1-enyl chains of total phospholipids include the glycerol-substituted alk-1-enyl chains in the glycerol acetals of the ethanolamine and *N*-methylethanolamine plasmalogens. These, like the ordinary alk-1-enyl chains, are released as aldehydes on acid hydrolysis. For acyl groups and alk-1-enyl groups, the number before the colon is the chain length and after, the number of double bonds. Cyc indicates a cyclopropane-containing chain.