Structure of the Membrane-Embedded F_0 Part of F_1F_0 ATP Synthase from *Escherichia coli* As Inferred from Labeling with 3-(Trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine†

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ABSTRACT: 3-(Trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID) is a photoactivatable carbene precursor designed to label selectively the hydrophobic core of membranes. We have used this reagent to obtain information on the topological organization of the membrane-embedded subunits of F_1F_0 ATP synthase from *Escherichia coli*. The study included [^{125}I]TID labeling of F_0 subunits in different structural (conformational) states and Edman degradations of the labeled polypeptides in order to assign the covalently bound radioactivity to individual amino acid residues. Released phenylthiohydantoin amino acids were analyzed by thin-layer chromatography, and the radioactive derivatives were visualized by autoradiography. The data suggest that labeling patterns can be correlated in a meaningful manner with reagent accessibility and hence with protein–lipid contact. Subunit b appears to be anchored to the membrane by a short N-terminal segment. As almost all of the amino acids of this part are accessible to the reagent, it is inferred that this segment has little interaction with the other subunits. In contrast, in the two segments of subunit c that were labeled with [^{125}I]TID, only certain amino acids reacted with the label. The pattern of these labeled residues is compatible with that of tightly packed α-helices.

ATP synthase from *Escherichia coli* is composed of two parts, F_1 and F_0: F_1 is peripheral and contains the ATP sites; F_0 is membrane embedded and catalyzes H^+ conduction across the membrane (Senior & Wise, 1983; Hoppe & Sebald, 1984). Recently, the nucleotide sequences of the genes coding for F_0 subunits a, b, and c have been determined, and thus the size and amino acid sequence of these subunits are known (Downie et al., 1981; Hansen et al., 1981; Negrin et al., 1980; Friedl & Schairer, 1981; Schneider & Altendorf, 1982; Nielsen et al., 1981; Kanazawa et al., 1981; Gay & Walker, 1981). Subunit a (M_r 30 267) is a protein that, from its high content of hydrophobic amino acid residues, is predicted to be largely embedded in the lipid phase of the membrane. Subunit b (M_r 17 212) is an amphiphilic protein, and subunit c (M_r 8288), also referred to as a proteolipid or dicyclohexylcarbodiimide-binding protein, is a highly hydrophobic component (Cattell et al., 1971). The latter has been identified in numerous organisms, and the amino acid sequences have been reported from nine species (Sebald et al., 1980; Hoppe & Sebald, 1980, 1982; Hoppe et al., 1980a,b; Sebald & Hoppe, 1981). A stoichiometry for a:b:c of 1:2:10–15 has been suggested (Foster & Fillingame, 1982; von Meyenburg et al., 1982).

The main goal of the present study was to derive information on the structure of the proton transducing moiety F_0 by labeling with the lipid-soluble, photoactivatable carbene generator [^{125}I]TID.[1] This compound is one of those reagents developed to generally label the lipid core of membranes (Chakrabarti & Khorana, 1975; Bercovici & Gitler, 1978; Bayley & Knowles, 1978; Hu & Wisnieski, 1979; Brunner et al., 1980; Brunner & Semenza, 1981; Bisson & Montecucco, 1981). Recent attempts to label the F_0 part of ATP synthase with aryl nitrene generating compounds have shown that essentially a single residue (Cys_{21} of the b subunit) could be derivatized by these reagents (Hoppe et al., 1983a,c). Thus, the absence of label in subunits a and c indicated a pronounced chemical selectivity of aryl nitrenes (or of other reactive intermediates generated therefrom) and demonstrates the necessity of using more reactive and hence less selective species for obtaining useful topological information. [^{125}I]TID has been shown to label in a highly specific manner those parts of intrinsic proteins that are embedded in the lipid bilayer; it remained uncertain, however, whether reactions with most inert, aliphatic amino acid side chains would occur to detectable extents. As many membranous segments of integral proteins are extraordinarily rich in aliphatic amino acids, this is a question of considerable importance with respect to choice of a reagent and interpretation of labeling data. In addition to our interest in the structure of F_0, the work described in the present paper represents also a start toward a more detailed characterization of [^{125}I]TID as a hydrophobic labeling reagent.

Materials and Methods

Bacterial Strains. Genotype of strain CM 2786 (CM 1470 (pBJC 706)) was used: CM 1470 *E. coli* K12 F$^+$ *asn B32 thi-1 rel A1 spo 11 atp 706* (del *atp I BEFHA*), *pBJC 706 atp* (BEFHAGDC)$^+$ on a dimer of pBR322. Expression of the ATP synthase genes on the plasmid is mainly due to transcription from a promotor in pBR322, resulting in ATP synthase levels 4–5-fold higher than in a wild-type strain.

Growth and Media. Cells were grown on Vogel and Bonner minimal medium (Vogel & Bonner, 1956). The carbon source was 1% glucose; ampicillin was added to 0.1 mg/mL. Cells

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[1] Abbreviations: [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; PTH, phenylthiohydantoin; INA, 5-[^{125}I]iodonaphthyl 1-azide; $NaDodSO_4$, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid.

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were grown to late log phase in 200-L cultures.

Preparation of ATP Synthase. The preparation of membranes, of F_1-depleted membranes, and of ATP synthase (F_1F_0) was as described (Friedl et al., 1979). The purity of F_1F_0 was at least 95%. Enzyme activities were identical with those described (Friedl et al., 1979).

Photolabeling. [^{125}I]TID was synthesized at a specific radioactivity of 10 Ci/mmol as described (Brunner & Semenza, 1981). The reagent (0.2–0.5 mCi in 10–20 μL of ethanol) was added to protein samples (see below) dissolved in 0.4–1 mL of buffer. Following incubation at room temperature for 15 min, samples were photolyzed by using an Aminco fluorometer equipped with a 250-W xenon lamp at 360 nm (bandwidth 40 nm) in a 1-cm quartz cuvette for 4 min at room temperature. During photolysis, samples were stirred with a small stirring rod. The samples used for labeling were as follows: (i) F_1F_0 ATP synthase, 2 mg of protein/400 μL of 50 mM Tris-HCl, 25 mM Aminoxid WS 35, 1 mM $MgCl_2$, 0.2 mM EGTA, 100 mM KCl, and 20% MeOH, pH 7.5; (ii) membranes prepared from the ATP synthase overproducing strain (these were adjusted to 6 mg of protein/mL of 1 mM Tris-HCl, 0.2 mM EDTA, and 5% glycerol, pH 7.5; (iii) F_1F_0 dissolved in $NaDodSO_4$ (purified F_1F_0 was precipitated with acetone and dissolved in 2% $NaDodSO_4$).

Separation of Subunits. Nine volumes of acetone (−20 °C) was added to [^{125}I]TID-labeled samples of F_1F_0. Precipitation of the protein was complete after 1 h at −20 °C. The precipitate was collected by brief centrifugation and dissolved in 0.2 M Tris–2% $NaDodSO_4$. Subunits were separated by high-performance gel permeation chromatography on a G 3000SW column in 200 mM sodium phosphate buffer, pH 7.0, containing 0.1% $NaDodSO_4$ (Hoppe et al., 1983a). Occasionally, rechromatography of the subunits was necessary. Purity was checked by $NaDodSO_4$–polyacrylamide gel electrophoresis, followed by autoradiography (Hoppe et al., 1983b).

Subunit b was isolated from labeled membranes by preparative $NaDodSO_4$ gel electrophoresis as described (Hoppe et al., 1983b). Subunit c was obtained from F_1-depleted membranes (from strain CM 2876) by extraction with chloroform–methanol, 2:1 (v/v), and filtration through a column (1 mL) of CM-52 (NH_4^+ form) equilibrated in chloroform–methanol, 2:1 (v/v). Proteolipid was not retained under these conditions and was pure as judged by $NaDodSO_4$ gel electrophoresis, followed by autoradiography.

Sequencing and Identification of Labeled Amino Acid Residues. Subunit b was immobilized on porous glass beads as described (Hoppe et al., 1983a). Subunit c (dissolved in 40% 2-chloroethanol, 1% $NaDodSO_4$, and 0.1 M pyridine hydrochloride, pH 5.0) was coupled to 70 mg of aminopropyl glass by using 4 mg of 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide and 4 mg of *N*-hydroxybenzotriazole as catalysts. This medium caused coupling mainly via the carboxy terminus of the protein. Edman degradation of this immobilized [^{125}I]TID-labeled protein generally results in an artifactual release of radioactivity from the glass beads whenever N-terminal Glu or Asp residues are cleaved. To circumvent this shortcoming, subunit c was immobilized via Lys_{34} to *p*-phenylenediisothiocyanate-activated glass in some experiments (Hoppe et al., 1983a).

Sequencing was performed as described (Hoppe & Sebald, 1979). PTH derivatives of amino acids were identified by TLC using solvent 1 [chloroform–ethanol, 98:2 (v/v)] as described (Hoppe & Sebald, 1979). Autoradiography of TLC plates was performed at −80 °C for 1–3 weeks; an intensifier screen was utilized. Radioactivity was determined by γ counting.

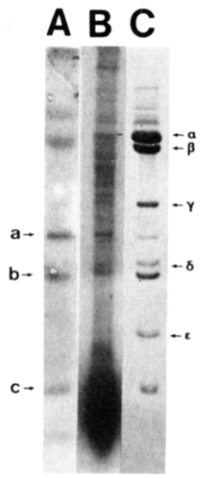

FIGURE 1: Labeling by [^{125}I]TID of subunits of F_1F_0 and F_1-depleted membranes. ATP synthase (F_1F_0) or F_1-depleted membranes were labeled with [^{125}I]TID as described (see Materials and Methods). Samples corresponding to approximately 10 μg of protein were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis. After being stained with Coomassie blue, the gel was dried and subjected to autoradiography using a Kodak X-O-Mat film. (A) F_1F_0 in Aminoxid WS 35 (autoradiogram); (B) F_1-depleted membranes (autoradiogram); (C) Coomassie blue stained gel of the purified F_1F_0 complex.

Peptide Analyses. CNBr cleavage, separation of peptides on Bio-Gel P-30 in 80% formic acid, and amino acid analyses were performed as described previously (Hoppe & Sebald, 1980).

Results

[^{125}I]TID Labels All Three Subunits of F_0. When purified F_1F_0 (in Aminoxid) or F_1-depleted membranes were labeled with [^{125}I]TID, all three subunits (a, b, and c) covalently incorporated radioactivity. This was demonstrated by autoradiography following protein separation by $NaDodSO_4$ gel electrophoresis (Figure 1) and after isolation of subunits b and c from labeled samples (see below). The extent of labeling was difficult to estimate because of the incomplete recovery of subunit c after acetone precipitation. But unlike labeling with aryl azide based reagents that gave rise to highly preferential labeling of subunit b, this subunit was less heavily labeled than subunit a and was labeled approximately to the same extent as polypeptide c (Hoppe et al., 1983a,c). Figure 1 also demonstrates that polypeptides α, γ, δ, and ϵ originating from F_1 were not or only barely labeled by [^{125}I]TID, which is consistent with the known preference of this reagent for labeling intrinsic membrane proteins (Brunner & Semenza, 1981). Throughout all our experiments with the hydrophobic photoactivatable reagents, we observed labeling of the F_1 subunit β. The reason for the labeling of this apparently peripheral protein is not clear, but it seems likely that it has some contact with the lipid bilayer or it contains hydrophobic regions on its surface that may bind some of the reagent. It has been noticed earlier that serum albumin, a water-soluble protein with a hydrophobic pocket on its surface, binds [^{125}I]TID (and other hydrophobic compounds) and is heavily labeled upon photolysis. Recently, it has been discovered that [^{125}I]TID also labels calmodulin and calmodulin fragments in a Ca^{2+}-dependent way (Krebs et al., 1984).

Distribution of Label among Individual Amino Acid Residues of F_0 Polypeptide Chains. Toward our goal, which is to derive a possible topological organization of the F_0 subunits, the general approach of hydrophobic photolabeling of membranes was extended. We performed labeling of F_0 subunits

in different states, and we used Edman degradation of [^{125}I]TID-labeled polypeptides in order to determine the individual residues modified by the reagent.

The systems used for [^{125}I]TID labeling were (i) F_1-depleted membranes (these represent the most native material), (ii) purified F_1F_0 in detergent micelles [labeling patterns were expected to be similar to those obtained in (i) since the structural organization of F_0 is not likely to be affected by either removal of F_1 or addition of detergent], and (iii) NaDodSO$_4$-denatured protein (although the subunits may still contain elements of intact structure, NaDodSO$_4$ solubilization should particularly affect reagent accessibility to subunit c, which exists as an oligomer in the native state and as a monomer in NaDodSO$_4$).

Protein chemical analysis of subunit a is difficult and only a few residues have so far been determined by Edman degradation (Hoppe et al., 1983b). The present detailed investigation was therefore restricted to subunits b and c. Nonetheless, the heavy labeling of subunit a by [^{125}I]TID (Figure 1) suggests that a large fraction of its surface is in contact with the apolar phase of the membrane.

Subunit b. To identify the regions of subunit b polypeptides that were accessible to the reagent in intact ATP synthase (F_1F_0–Aminoxid WS 35), the purified subunit was cleaved with CNBr. Three peptides resulted, which were readily separated by gel filtration on Bio-Gel P30 using 80% formic acid (data not shown). More than 98% of the radioactivity was associated with two small N-terminal peptides (residues 2–22 and 23–30), indicating that only this region is in contact with the membranous lipid phase.

For the determination of the modified amino acid residues, the whole subunit b was subjected to Edman degradation and the radioactivity, eluted at each cycle, was monitored (Figure 2B). Edman sequencing was also performed with subunit b isolated upon labeling under different conditions (systems i–iii). Figure 2 shows that, in all cases, Cys_{21} was the dominant site of labeling. However, there were additional degradation cycles during which radioactivity was released and which indicate labeling of less reactive residues by the carbene. To examine this point, PTH amino acid derivatives were subjected to TLC and plates were autoradiographed to detect the radioactive components. As shown in Figure 3, radioactivity patterns were obtained that appear to be characteristic for a given amino acid residue (e.g., compare Phe_{14}, Phe_{17}, and Phe_{20}). The existence of such patterns indicates that the radioactivity released was in fact due to labeled amino acid residues and not of artificial origin (for example, due to loss of protein or "carry-over" from a preceding heavily labeled residue). Clearly, the aliphatic residues (i.e., valine, leucine, and isoleucine) were labeled. Thus, from these data we conclude that [^{125}I]TID was capable of labeling a wide range of amino acid residues, which also includes those containing aliphatic side chains.

Subunit c. Labeled amino acid residues of this subunit were determined by sequencing the whole polypeptide chain (Figure 4). By using the optimized procedure for immobilization of the protein (Materials and Methods), it was possible to identify all 79 amino acid residues as their PTH derivatives by TLC. Attempts to obtain CNBr fragments of labeled subunit c were unsuccessful because modification of methionyl residues prevented cleavage at these sites. A complex mixture of radioactive peptides resulted that could not be separated by Bio-Gel P30 gel filtration for subsequent identification.

While similar radioactivity distribution patterns were obtained for subunit c labeled either in F_1-depleted membranes

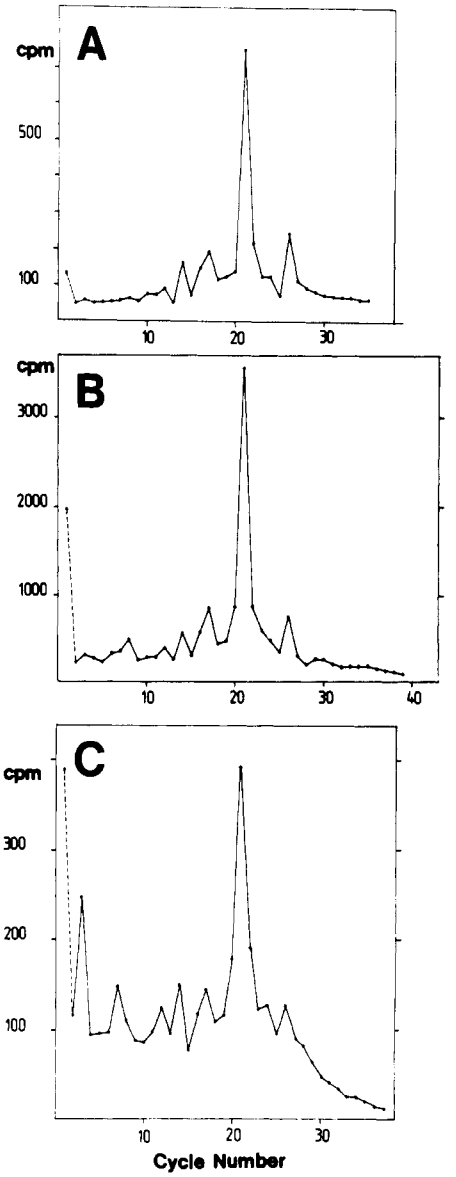

FIGURE 2: Distribution of [^{125}I]TID label among individual amino acid residues in the N-terminal region of subunit b. [^{125}I]TID labeling and isolation of subunit b were performed as described. Protein containing between 30 000 and 60 000 cpm was immobilized on *p*-phenylenediisothiocyanate-activated porous glass beads and subjected to Edman degradation. PTH amino acids were analyzed for iodine-125 radioactivity. (A) Subunit b labeled in NaDodSO$_4$-denatured F_1F_0; (B) subunit b labeled in intact F_1F_0 (in Aminoxid WS 35); (C) subunit b labeled in F_1-depleted membranes.

(i) or in intact F_1F_0 (ii) (Figure 5B,C), a completely different pattern resulted upon labeling in the NaDodSO$_4$-dissociated form (Figure 5A). In the intact subunit, labeling was confined to a few sites that lie within two regions located near the N- and C-termini of the polypeptide chain; in the NaDodSO$_4$-denatured state (iii), labeling was spread over the entire chain and a large number of residues were modified by the carbene.

In all histograms of Figure 5, the sites of dominant labeling are methionyl residues that evidently have a high intrinsic reactivity toward the carbene. However, in spite of this obvious preference of the reagent for labeling sulfur-containing centers (Cys in subunit b and Met in subunit c), it is important to notice that different sets of methionines were labeled in intact F_0 [(i) and (ii)] and in NaDodSO$_4$-dissociated form (iii). This suggests that labeling patterns are primarily determined by the reagent accessibility and *not* by the intrinsic reactivity of individual amino acid residues.

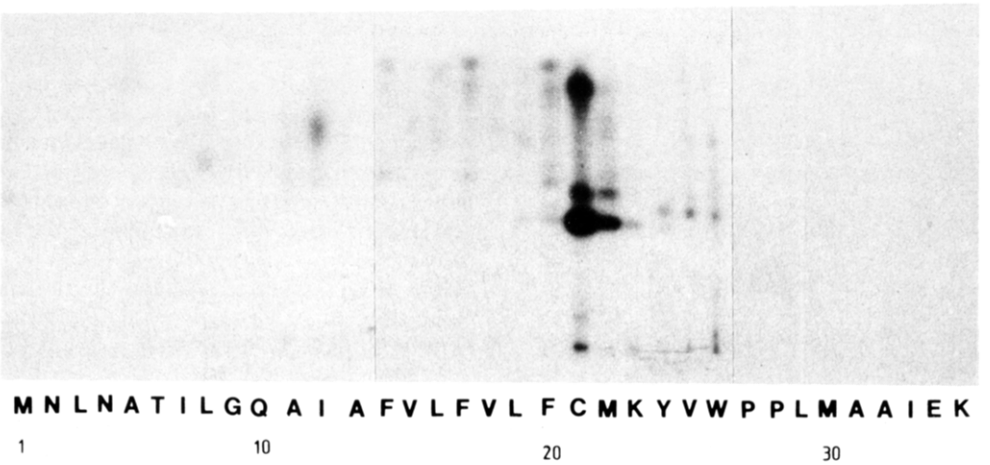

FIGURE 3: Autoradiogram demonstrating the TLC patterns of the radioactive components (PTH derivatives) obtained from Edman sequencing cycles of subunit b polypeptide that had been isolated from [^{125}I]TID-labeled F_1F_0.

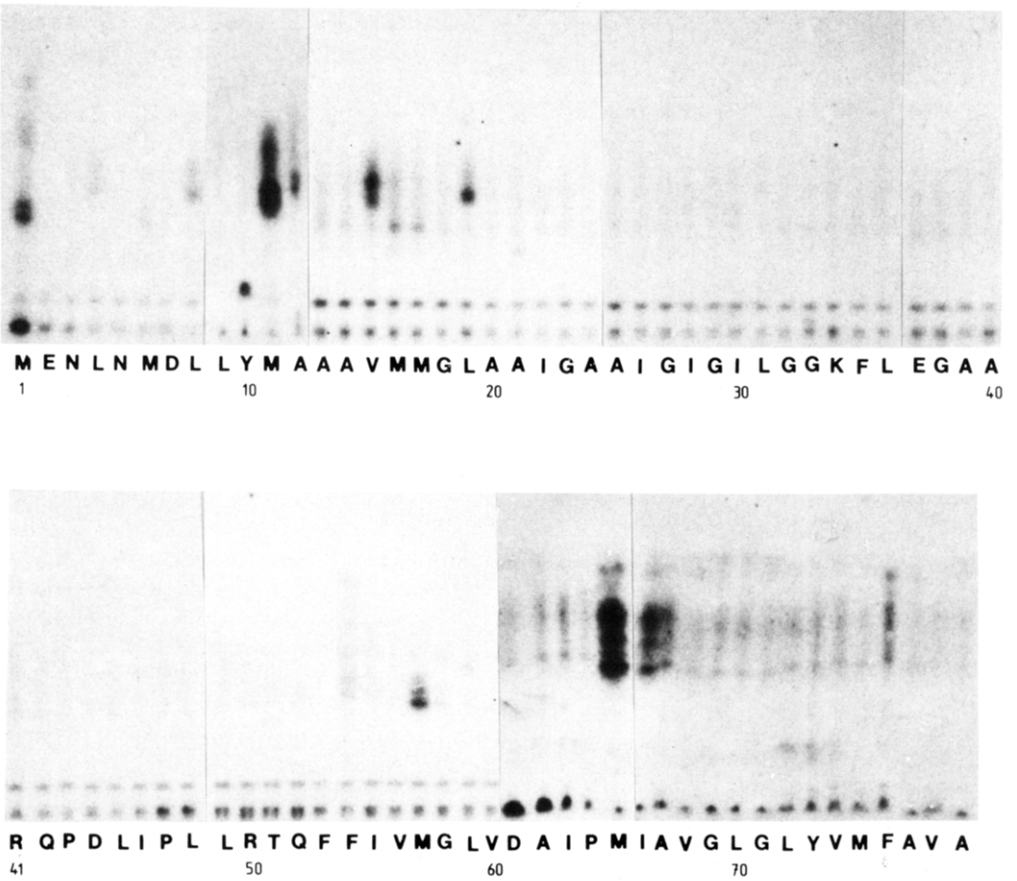

FIGURE 4: Autoradiogram demonstrating the TLC patterns of the radioactive components (PTH derivatives) obtained from Edman sequencing of subunit c polypeptide isolated from [^{125}I]TID-labeled F_1-depleted membranes. Increasing fractions of the total PTH amino acid samples ($^1/_{10}$ for cycles 1–26, $^1/_4$ for cycles 27–49, and $^1/_2$ for cycles 50–80) were applied onto the TLC plates to compensate for decreasing Edman degradation yields.

As even carbenes exhibit some chemical selectivity, it is not surprising that labeling of nucleophilic amino acid side chains was, in general, more efficient than that of other residues. However, the present data strongly suggest that essentially all amino acid residues could be modified to extents that did not escape detection by the analytical procedures used here. So far, no labeling of alanyl residues has been observed, which can be explained by the very low reactivity of the carbene for primary C–H bonds (F. M. Richards, personal communication). Likewise, we did not observe modification of Glu and Asp side chains although the carboxyl group should be readily esterified by the carbene (Ross et al., 1982). We therefore assume that esters were formed but were cleaved again during subsequent treatment(s) of the protein with acid (deformylation in MeOH–HCl). Clearly, more data will be required to clarify the photoreaction of [^{125}I]TID with carboxyl groups and to determine the chemical properties of the products obtained thereby.

Discussion

In an attempt to correlate the labeling of F_0 subunits b and c with a possible topological arrangement of the polypeptide chains within the lipid bilayer, some of the considerations taken into account are as follows: First, as shown previously for

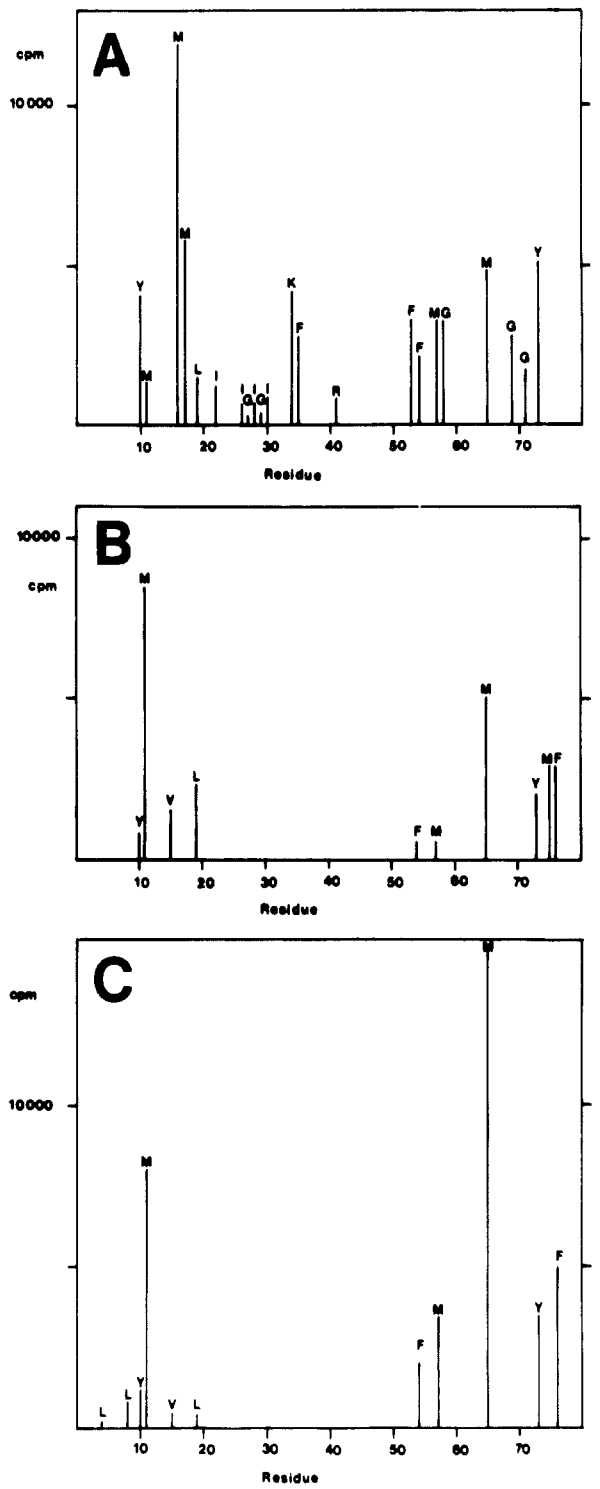

FIGURE 5: Histograms of the distribution of iodine-125 radioactivity among individual amino acid residues of subunit c polypeptide. The protein was isolated upon [^{125}I]TID labeling of (A) subunit c in $NaDodSO_4$ solution, (B) F_1F_0 in Aminoxid WS 35, and (C) F_1-depeleted membranes. The figures (cpm) were corrected for decreasing yields during Edman degradation, assuming a repetitive yield of 95%.

proteins with known topology (glycophorin, sucrase–isomaltase, and others), [^{125}I]TID labeling of intrinsic proteins was confined to those polypeptide segments that are buried in the lipid bilayer and was below detection for peripheral domains (Brunner & Semenza, 1981; Brunner et al., 1983; Spiess et al., 1982). However, in some instances [^{125}I]TID may also bind to hydrophobic pockets in water-soluble proteins (Krebs et al., 1984). On the basis of the [^{125}I]TID labeling data, it is not possible to distinguish whether the labeling of the F_1 subunit β occurred from the lipid phase or by a binding of [^{125}I]TID at a hydrophobic pocket located in the peripheral domain. Clearly, [^{125}I]TID labeling data will have to be supported by experiments using different types of reagent that are not able to interact with peripheral domains (e.g., diazirines attached to lipids). It should be noted that a photoreactive lipid bearing a nitrene precursor also labeled the F_1 subunit β (Hoppe et al., 1983a), favoring the model that this subunit has some contact with lipids. For the [^{125}I]TID labeling experiments it thus appears that besides this unexpected reaction with subunit β, labeling is restricted to the membrane-integrated F_0 proteins of the ATP synthase. This suggests that amino acid residues that received label are likely to be in contact with the membranous lipid core. Second, in the following analysis we do not take into account the existing differences in the intrinsic reactivities of the individual amino acid residues toward the carbene or any possible anisotropy in the distribution of reagent at the protein–lipid interface. On the basis of these simple assumptions and approximations, the Edman degradation data have been used to suggest models for the assembly of subunits b and c within the membrane. The main features of these models are discussed below.

Subunit b. This subunit appears to be anchored in the membrane by a short N-terminal segment only. This stretch, possibly in an α-helical conformation (van Heijne, 1981; Engelman & Steitz, 1981), starts at the very beginning of the polypeptide chain and ends at position 27 or 28, where two prolines are located. These residues would interrupt the hydrogen bonding of the α-helix, thereby generating a hydrophilic area that most likely would not be integrated into the membrane.

Many residues in this putative α-helical segment are labeled, indicating little contact with other polypeptide chains. Clearly, one must also consider the possibility that the two copies of subunit b polypeptide present per F_0 are arranged asymmetrically, thereby exposing different surface sectors to the lipid phase. The present data do not allow one to strictly distinguish between these possibilities, but due to the close similarities of the radioactivity profiles obtained with the native enzyme and freely accessible monomeric subunits in an $NaDodSO_4$ solution (Figure 2A,B), we favor the model of free reagent accessibility to segment 12–21 in subunit b. The absence of label in Lys_{23} and Tyr_{24} may indicate that these residues are part of a protein–protein contact area. Another contact region might include Asn_2, Thr_6, and Gln_{10}, which were not labeled and would reside on the same side of the helix.

Recently, Khorana and associates have applied diazirine-containing phospholipids in an attempt to elucidate the mode of association of cytochrome b_5 with the phospholipid bilayer (Takagaki et al., 1983a,b). The structure of cytochrome b_5 resembles that of subunit b in that only a short segment of the protein is anchored in the membrane. Similarly to the results obtained with [^{125}I]TID-labeled subunits b, a broad bell shape like distribution of cross-linked phospholipid was found in the segment Ser_{104}–Met_{130} of cytochrome b_5, indicating a high accessibility of the membrane segment to the generated carbenes.

Subunit c. Labeling of subunit c is strikingly different from that of subunit b as only discrete amino acids were labeled. As inferred from the labeling of these residues, there is extended contact among membrane-embedded segments of subunit c. Interestingly, those residues of the N-terminal segment that were labeled in the intact F_0 are distributed in such a way that they would all lie on the same side of an α-helix (Figure 6). Radioactivity patterns in which consec-

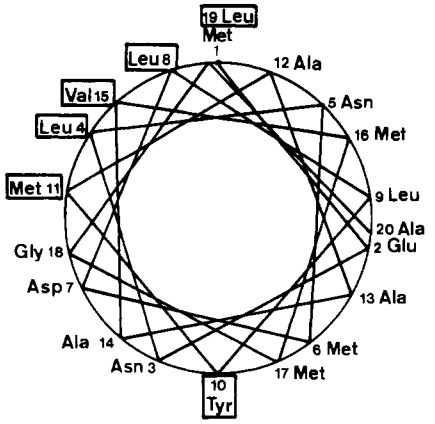

FIGURE 6: Axial projection of the N-terminal segment of the polypeptide c from *E. coli* ATP synthase in an α-helical structure. Amino acid residues in boxes are those labeled by [^{125}I]TID.

utive labeled residues appear in a sequence with an average periodicity of three to four residues may therefore be indicative (a) of an α-helical conformation of the respective polypeptide segment and (b) of tight packing, exposed only a fraction of the helix surface to the lipid phase. Phe_{54}–Met_{57} may also be located in an α-helix, which would probably be interrupted at Pro_{64}. A third helical segment may start at Met_{65} as indicated by the labeling of Met_{65}, Phe_{70}, and Tyr_{73}, which again would lie on the same side of a helix. It is remarkable that almost identical α-helical regions were postulate from CD measurements in conjunction with secondary structure predictions (Sebald & Hoppe, 1981).

The absence of label within the segment ranging from residues 34 to 52 provides supporting experimental evidence to another study proposing that this part of the polypeptide chain extends from the lipid phase into the cytoplasm (Sebald & Hoppe, 1981).

Although, as inferred from its hydrophobicity, the glycine-rich, conserved segment from Ala_{20} to Gly_{33} would be predicted to be embedded in the lipid bilayer, no labeling of this segment was obtained in $NaDodSO_4$ solution. This observation is interpreted to suggest that this region is located within the membrane but shielded by other polypeptide segments from the lipid bilayer. In this case, the lipid core would not exert an α-helix-promoting force on this segment, which, as predicted, may adopt a β-sheet conformation (Sebald & Hoppe, 1981). It is possible that, in the oligomeric complex, these sheets are assembled to a β-barrel, which is a common structural element of many proteins. For example, β-barrel structures formed by eight parallel strands have recently been reviewed by Muirhead (1983).

Quaternary Structure. One important conclusion regarding the quaternary structure of the F_0 subunits within the membrane may be drawn from the [^{125}I]TID-labeling experiments. Since all subunits are highly accessible to the reagent, it seems unlikely that subunits a or b are located inside a core formed by the 10 copies of subunits. For the N-terminus of subunit b, these findings have been substantiated by results from labeling studies with nitrene-generating phospholipids (Hoppe et al., 1983a). It seems more likely that the three subunits are arranged in a highly unsymmetric fashion.

Acknowledgments

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Registry No. [^{125}I]TID, 79684-41-6; ATP synthase, 37205-63-3; TID, 81340-56-9.

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Topology of Beef Heart Cytochrome *c* Oxidase from Studies on Reconstituted Membranes†

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ABSTRACT: The orientation of purified beef heart cytochrome *c* oxidase, incorporated into vesicles by the cholate dialysis procedure [Carroll, R. C., & Racker, E. (1977) *J. Biol. Chem. 252*, 6981], has been investigated by functional and structural approaches. The level of heme reduction obtained by using cytochrome *c* along with the membrane-impermeant electron donor ascorbate was 78 ± 2% of that obtained with cytochrome *c* and the membrane-permeant reagent *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine. Electron transfer from cytochrome *c* is known to occur exclusively from the outer surface of the mitochondrial inner membrane (C side), implying that at least 78% of the oxidase molecules are oriented in the same way in these vesicles as in the intact mitochondria. Trypsin, which cleaves subunit IV near its N terminus, modifies only 5–7% of this subunit in intact vesicles. This removal of the N-terminal residues has been shown to occur only in mitochondrial membranes with their inner side (M side) exposed. Diazobenzene[^{35}S]sulfonate ([^{35}S]DABS) likewise modifies subunit IV only in submitochondrial particles. Labeling of intact membranes with [^{35}S]DABS resulted in incorporation of only 4–8% of the total counts that could be incorporated into this subunit in membranes made leaky to the reagent by addition of 2% Triton X-100. Therefore, both the functional and structural data show that at least 80% and probably more of the cytochrome *c* oxidase molecules are oriented with their C domain outermost and M domains in the lumen of vesicles prepared by the cholate dialysis method. Labeling experiments with [^{35}S]DABS and proteinase digestions of intact membranes with trypsin and chymotrypsin were used to determine the topography of the subunits of cytochrome *c* oxidase. These studies confirm that subunits II and III are in the C domain and subunits IV and VII in the M domain and show for the first time that polypeptides b and c are on the C side of the mitochondrial inner membrane.

The exposure of the subunits of cytochrome *c* oxidase at different sides of the mitochondrial inner membrane has been probed by labeling with membrane-impermeant protein-modifying reagents such as diazobenzenesulfonate (DABS)[1] (Schneider et al., 1971; Eytan et al., 1975; Ludwig et al., 1979; Prochaska et al., 1980), by digestion of components with proteases (Malatesta et al., 1983a), and by reaction with antibodies raised to individual subunits of the enzyme (Chan & Tracy, 1978).

These studies, taken together, show cytochrome *c* oxidase as a transmembrane protein with most of the mass of subunit IV located on the matrix (M) side of the inner membrane, with most of subunit II on the cytoplasmic (C) side, and with subunits I, II, III, IV, and VII(s) [see Capaldi et al. (1983b) for nomenclature] each spanning the lipid bilayer at least once.

Recently, the sequences of all of the subunits of beef heart cytochrome *c* oxidase have been obtained either by direct procedures or by sequencing of mDNA [e.g., see Steffens et al. (1979), Tanaka et al. (1975), and Anderson et al. (1982)]. This makes possible more detailed topological studies at the level of the folding of individual polypeptides.

Detailed analysis of the folding of subunits in cytochrome *c* oxidase is difficult when the enzyme is labeled or digested by proteases in mitochondrial membranes because of the problems inherent in isolating modified proteins. For example, it requires purifying cytochrome *c* oxidase from large volumes of highly radioactive mitochondria in order to have sufficient enzyme with high enough specific activity for subunit isolation and fragmentation experiments. Also, chemically modified cytochrome *c* oxidase or enzyme partly digested by proteases may not purify in a manner identical with that of the native enzyme.

The folding of individual subunits of cytochrome *c* oxidase should be amenable to experimentation in vesicle preparations containing purified enzyme and phospholipid, provided that

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[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; DCCD, dicyclohexylcarbodiimide; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; DABS, diazobenzenesulfonate; PMSF, phenylmethanesulfonyl fluoride; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; Hepes, *N*-(2-hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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