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F_o Portion of *Escherichia coli* ATP Synthase: Orientation of Subunit c in the Membrane†

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ABSTRACT: Incubation of right-side-out oriented membrane vesicles of *Escherichia coli* with tetranitromethane resulted in the nitration of tyrosine residues (Tyr-10 and Tyr-73) of subunit c from the ATP synthase. Cleavage of the protein with cyanogen bromide and separation of the resulting fragments, especially of the tyrosine-containing peptides, clearly demonstrated that the distribution of the nitro groups is similar at any time and at any pH value chosen for the analysis. Furthermore, the percentage of 3-nitrotyrosine present in the two peptide fragments was in good agreement with that obtained for the intact polypeptide chain. While the modification of the tyrosine residues in subunit c with the lipophilic tetranitromethane is independent of the orientation of the membrane vesicles, the subsequent partial conversion of the 3-nitrotyrosine to the amino form only occurred when membrane vesicles with right-side-out orientation were treated with the ionic, water-soluble sodium dithionite, which at certain concentrations cannot penetrate biological membranes. Cleavage of subunit c isolated from nitrated and subsequently reduced membrane vesicles and separation of the resulting fragments by high-pressure liquid chromatography showed that the 3-nitrotyrosine in the Tyr-73-containing peptides has been completely reduced, while the nitro group in peptides containing Tyr-10 remained nearly unaffected.

The membrane-bound proton-translocating ATP synthase (F_1F_o) of *Escherichia coli* catalyzes the synthesis of ATP by utilizing energy of an electrochemical proton gradient built up by respiration. The enzyme can also function in the reverse direction by coupling hydrolysis of ATP to proton translocation across the membrane, thereby generating an electrochemical gradient of protons essential for driving, e.g., active transport (Senior & Wise, 1983; Futai & Kanazawa, 1983).

The enzyme complex consists of two structurally and functionally distinct entities designated F_1 and F_o. The water-soluble F_1 part consisting of five different polypeptides (α, β, γ, δ, ϵ) with a stoichiometry of 3:3:1:1:1, respectively, carries the catalytic and regulatory centers of the enzyme (Vignais & Satre, 1984). F_1 is bound by electrostatic and hydrophobic interactions to the membrane-integrated part, F_o, which is built up of three kinds of subunits (a, b, c) and functions as a H^+ channel (Hoppe & Sebald, 1984; Senior, 1985; Walker et al., 1984). The stoichiometry of the F_o subunits is proposed to be 1:2:10 ± 1 for a:b:c, respectively (Foster & Fillingame, 1982). The analysis of mutant strains lacking one of the three F_o subunits (Friedl et al., 1983) and

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reconstitution experiments with isolated subunits a, b, and c (Schneider & Altendorf, 1985) indicate that all three proteins are necessary to obtain an F_o complex functional in proton translocation and binding of F_1.

The amino acid sequences of the F_o subunits have been deduced from DNA sequencing data (Walker et al., 1984). In addition, the primary structure of subunit c has first been established by Edman degradation (Sebald & Wachter, 1978). Subunit b is a rather hydrophilic protein with only a hydrophobic segment at its N-terminal region, which anchores the protein in the membrane. The hydrophilic part is exposed to the cytoplasm and highly susceptible to proteases (Perlin & Senior, 1985; Steffens et al., 1987). Subunit a is an extremely hydrophobic protein that may span the membrane 5 times, probably leaving the relatively hydrophilic N-terminal region exposed to the water phase (Senior, 1983; Hoppe & Sebald, 1984). Immunological studies with antibodies raised against subunit a indicate that the polypeptide chain is partially accessible from both sides of the membrane (Deckers-Hebestreit & Altendorf, 1986).

The amino acid sequence of subunit c[1] exhibits that the protein consists of two hydrophobic stretches separated by a polar segment with high turn potential. This clustering of polar and apolar residues, which is conserved not only in other bacteria but also in mitochondria and chloroplasts, suggests that the protein traverses the membrane twice in a hairpin-like structure (Sebald & Hoppe, 1981; Senior, 1983). Labeling experiments with the hydrophobic carbene generator TID[2] indicate that both hydrophobic regions are located in the lipid bilayer (Hoppe et al., 1984). Analyses of mutant strains, like DCCD-resistant mutants, in which Ile-28 is exchanged by either valine or threonine (Wachter et al., 1980; Hoppe et al., 1980), or a partial revertant strain, in which a second mutation (Ala-20 → Pro) partially suppresses the effects of the Pro-64 → Leu mutation (Fimmel et al., 1983), also corroborate the U-shaped conformation of subunit c. Studies with antibodies against subunit c reveal that the protein is accessible from both sides of the cytoplasmic membrane (Loo et al., 1983; Deckers-Hebestreit & Altendorf, 1986; Deckers-Hebestreit et al., 1986).

In this paper we have investigated the orientation of subunit c in the membrane by chemical modification. Due to their spatial distance in the polypeptide chain, the two tyrosine residues (Tyr-10 and Tyr-73) are ideal candidates for such labeling studies. The accessibility of these amino acid residues has been probed by use of membrane vesicles with different orientation and reagents different in hydrophobicity.

Experimental Procedures

Materials. Chromatography media and chemicals were purchased from the following companies: CM-cellulose (CM 23) from Serva (Heidelberg); HPLC column Varian MCH-10 (0.4 × 30 cm) from Varian (Hamburg) and Lichrosorb RP 18 column (5 μm; 0.4 × 25 cm) from Merck (Darmstadt); acetonitrile, trifluoroacetic acid, phosphoric acid (all especially purified for spectroscopic purposes), and sodium dithionite from Merck (Darmstadt); tetranitromethane (no longer available) and cyanogen bromide from Serva (Heidelberg). All other chemicals were of analytical grade.

Bacterial Growth. The *Escherichia coli* strain ML 308-225 ($i^-z^-y^+a^+$) was grown in the minimal medium of Davis and Mingioli (1950) with 0.4% glucose as energy source.

Preparation of Membrane Vesicles. Preparation of membrane vesicles with right-side-out orientation was carried out by the EDTA/lysozyme method described by Kaback (1971) from frozen cells harvested in late logarithmic phase. Membrane vesicles with inside-out orientation were also prepared from frozen cells as stated by Vogel and Steinhart (1976). For the depletion of F_1, the membrane vesicles were washed once in 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 10% (v/v) glycerol and incubated overnight at 4 °C in 1 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, and 10% (v/v) glycerol (protein concentration ~5 mg/mL). After centrifugation (90 min, 200000*g*), the incubation was repeated twice for 1 h at 4 °C. After this procedure the remaining ATPase activity of the everted membrane vesicles was lower than 2% of the starting activity.

Modification of Tyrosine Residues in Subunit c. The nitration of tyrosine residues in proteins with tetranitromethane was performed according to experiments described by Riordan and Vallee (1972) and Sone et al. (1979). Membrane vesicles were resuspended in 0.1 M Tris-HCl, pH 8.0, with a protein concentration of 2 mg/mL (for right-side-out oriented vesicles) or 6 mg/mL (for F_1-depleted everted vesicles) and incubated with 15 mM (for right-side-out oriented vesicles) or 20 mM tetranitromethane (for F_1-stripped everted vesicles) for 4 h at 25 °C. In membrane vesicles with right-side-out orientation, the outer membrane and the murein layer have been mostly removed by incubation with lysozyme and EDTA. In contrast, F_1-depleted everted membrane vesicles were prepared from intact cells with the Ribi press. Therefore, these vesicles contain many protein components of the outer membrane in their interior. Taking this additional protein into account, the protein concentration used has been increased compared to that adjusted for right-side-out vesicles. The conditions described were necessary for a complete nitration of the tyrosine residues of subunit c. Other conditions used were indicated in the figure legends. The reaction was stopped with 140 mM 2-mercaptoethanol, and the membrane vesicles were washed twice in the incubation buffer. For the reduction of 3-nitrotyrosine residues to the amino form with sodium dithionite (Sokolovsky et al., 1967), nitrated membrane vesicles were resuspended in 0.1 M potassium phosphate buffer, pH 6.6, with the same protein concentration as described above. After incubation with $Na_2S_2O_4$ (5 or 10 mM) for 15 min at 25 °C, the reaction was stopped with 50 mM hydrogen peroxide. The membranes were washed twice with the incubation buffer, and subsequently, subunit c was isolated.

Isolation and Cleavage of Subunit c. For the extraction of subunit c, membrane vesicles were treated with chloroform/methanol as described by Fillingame (1976) and Altendorf (1977). For the isolation of larger amounts of unlabeled proteolipid, whole cells were used as starting material (Altendorf et al., 1979). The resulting crude subunit c was purified to homogeneity by chromatography with the ion exchanger CM-cellulose (CM 23) in the presence of chloroform/methanol (Graf & Sebald, 1978; Altendorf et al., 1979). Cleavage of subunit c with cyanogen bromide was carried out in 80% (v/v) aqueous formic acid as described by Sebald et al. (1980).

Separation of CNBr Fragments of Subunit c. The dried peptide mixture obtained after CNBr treatment was dissolved in 80% (v/v) aqueous formic acid and a sample applied to a reverse-phase C_{18} column on a Gynkotech high-pressure liquid

[1] Subunit c is also referred to as proteolipid or DCCD-binding protein.

[2] Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

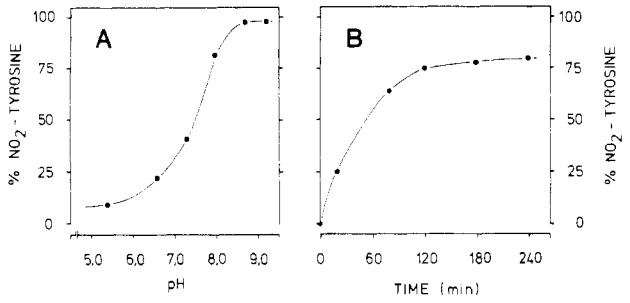

FIGURE 1: Nitration of the tyrosine residues of subunit c in right-side-out oriented membrane vesicles with tetranitromethane as a function of pH and time. Right-side-out vesicles were treated with 10 mM tetranitromethane for 4 h at the pH values indicated (A) or at pH 8.0 for the times indicated (B). After isolation of subunit c from those membranes, the degree of nitration was determined by amino acid analysis. At pH values above 7.0 the nitration was performed in 0.1 M Tris (adjusted with HCl), while at pH values below 7.0 a potassium phosphate buffer (0.1 M) was used.

chromatograph. With a Varian MCH-10 column, the peptides were eluted as described by Fimmel et al. (1985), and with a Lichrosorb RP 18 column, the peptides were separated as stated by Hoppe et al. (1982). The elution was monitored by measuring absorption at 210, 280, and 360 nm. Peak fractions were collected, dried, dissolved in 6 N HCl, hydrolyzed, and analyzed for amino acids.

Quantitation of Degree of Nitration or Reduction of Tyrosine Residues in Subunit c. The nitration and reduction rates of the tyrosine residues of subunit c were quantitated by amino acid analysis. 3-Nitrotyrosine eluted as a discrete peak immediately after phenylalanine. The amount of 3-nitrotyrosine has been calculated with the ninhydrin color factor of tyrosine, since the decrease of the tyrosine peak was always equivalent to the increase of the 3-nitrotyrosine peak. Quantitations of the hydrolyzed model compound *N*-acetyl-3-nitrotyrosine by amino acid analyses were also in good agreement with these calculations (data not shown). In contrast to 3-nitrotyrosine, 3-aminotyrosine is unstable during acid hydrolysis. For this reason the degree of nitrotyrosine reduction has been determined indirectly from the loss of 3-nitrotyrosine.

Analytical Methods. Protein was determined with bovine serum albumin as standard by the method of Lowry et al. (1951). In the case of membrane vesicle preparations the modification of Hartree (1972) and for subunit c isolated with chloroform/methanol the modification of Fillingame (1975) were applied. The F_1 ATPase activity was analyzed with the continuous phosphate detection system of Arnold et al. (1976).

For determination of amino acid compositions, proteins or CNBr-derived peptides (10–20 μg) were hydrolyzed under vacuum in 0.3 mL of 6 N HCl for 48 h at 110 °C and analyzed on a LC 5000 amino acid analyzer (Biotronik, München) with a BTC 2710 resin or on a BC 200 amino acid analyzer (LKB, München) with a cation exchanger from Durrum (type DC6).

RESULTS

Nitration of Tyrosine Residues of Subunit c. The reaction of tetranitromethane with tyrosine residues results in the introduction of a nitro group into the aromatic ring ortho to the phenolic hydroxyl that has to be in a deprotonated state (Sokolovsky et al., 1966). Due to its strong lipophilic nature, tetranitromethane is insoluble in aqueous solutions (Sone et al., 1979) and thought to accumulate in a hydrophobic environment.

Membrane vesicles with right-side-out orientation were incubated with tetranitromethane as a function of pH and time (Figure 1). After termination of the reaction, subunit c was isolated by chloroform/methanol extraction and analyzed for its amino acid composition. The percentage of 3-nitrotyrosine increased with the length of incubation time and after 4 h under the conditions used resulted in a nitration of 80% of the tyrosine residues (Figure 1B). Therefore, both residues present in subunit c were modified by tetranitromethane. The incubation of right-side-out vesicles at different pH values showed a similar pH-dependent nitration of subunit c (Figure 1A) as observed with model compounds (like *N*-acetyltyrosine) in aqueous solutions (Sokolovsky et al., 1966). However, at pH values above 8.0 the vesicular structure of the membranes is no longer guaranteed. Nevertheless, incubation with tetranitromethane was also carried out at alkaline pH values, since the nitration rate is always independent of the orientation of membrane vesicles (see Table II) due to the good solubility of tetranitromethane in the lipid bilayer.

Table I: Distribution of 3-Nitrotyrosine in CNBr Peptides B3 and B8 Derived from Subunit c after Time-Dependent Nitration of Right-Side-Out Vesicles with Tetranitromethane at pH 8.0[a]

	peptide B3		peptide B8	
time (h)	Tyr (%)	NO_2-Tyr (%)	Tyr (%)	NO_2-Tyr (%)
0	50		50	
0.5	35	15	35	15
1.0	17	33	22	28
2.0	9	41	2	48
3.0	5	45	7	43
4.0	10	40	9	41

[a] The percentage of the tyrosine- or 3-nitrotyrosine-containing peptides present at each time was calculated from the peak areas of the corresponding HPLC elution profiles (see Figure 2). The average values were determined from chromatograms measured at wavelengths of 210 and 280 nm, taking into account the nearly threefold higher molar absorptivity of the 3-nitrotyrosine at 280 nm compared to the tyrosine absorption (Sokolovsky et al., 1966).

The nitration rates in Figure 1 were determined for both tyrosine residues of subunit c. The cleavage of the polypeptide chain with cyanogen bromide and the separation of the resulting fragments, especially of the tyrosine-containing peptides B3 (residues 7–11) and B8 (residues 66–75), on a C_{18} reverse-phase HPLC column allowed a discrimination between the degree of nitration in Tyr-10 and Tyr-73. As shown in Figure 2, the peaks of both tyrosine-containing peptides disappeared as a function of time, while simultaneously the absorption of two 3-nitrotyrosine-containing peaks (identified by amino acid analysis, not shown) increased. Due to the high molar absorptivity of 3-nitrotyrosine compared to tyrosine at 280 nm (Sokolovsky et al., 1966), the increase in absorption of B3-NO_2 and B8-NO_2 is much stronger than the decrease of the unmodified peptides. After time-dependent nitration, the distribution of 3-nitrotyrosine in peptides B3 and B8 was determined by calculating the peak areas of these peptides from the corresponding HPLC chromatograms. The values presented in Table I reflected the same degree of nitration for each separated tyrosine residue of subunit c as has been determined for the intact protein. Similar results have been obtained after pH-dependent nitration (data not shown).

Nitration and Subsequent Reduction of Tyrosine Residues of Subunit c. In membrane vesicles both tyrosine residues of subunit c could be nitrated with the lipophilic reagent tetranitromethane. Whether both residues are completely embedded in the lipid bilayer of the membrane or whether they are also accessible from the membrane surface has been investigated with the ionic, water-soluble sodium dithionite. In the concentration range used the dithionite anion cannot penetrate the membrane (as can be deduced from the data

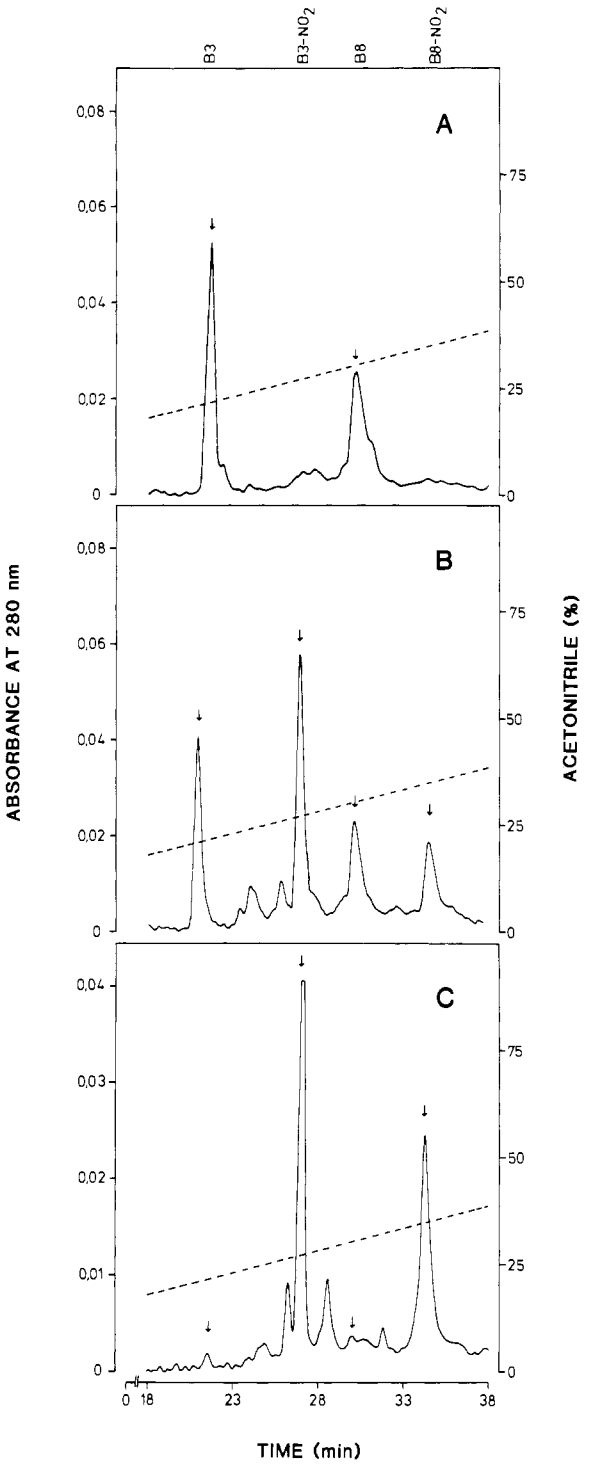

FIGURE 2: High-pressure liquid chromatography of peptides from CNBr-treated subunit c nitrated for different lengths of time: (A) untreated subunit c; (B) subunit c after nitration for 0.5 h; (C) subunit c after nitration for 4.0 h. Subunit c modified and isolated as described in the legend to Figure 1B was cleaved with CNBr. The obtained peptides were separated on a Varian MCH-10 reverse-phase column with an acetonitrile gradient in 0.1% aqueous phosphoric acid and identified by amino acid analysis (not shown). The absorption of the tyrosine-containing peptides was measured at a wavelength of 280 nm. The dashed line indicates the acetonitrile gradient used for the elution of the fragments. Different amounts of protein were applied to the column. The peptides were designated as described by Wachter et al. (1980).

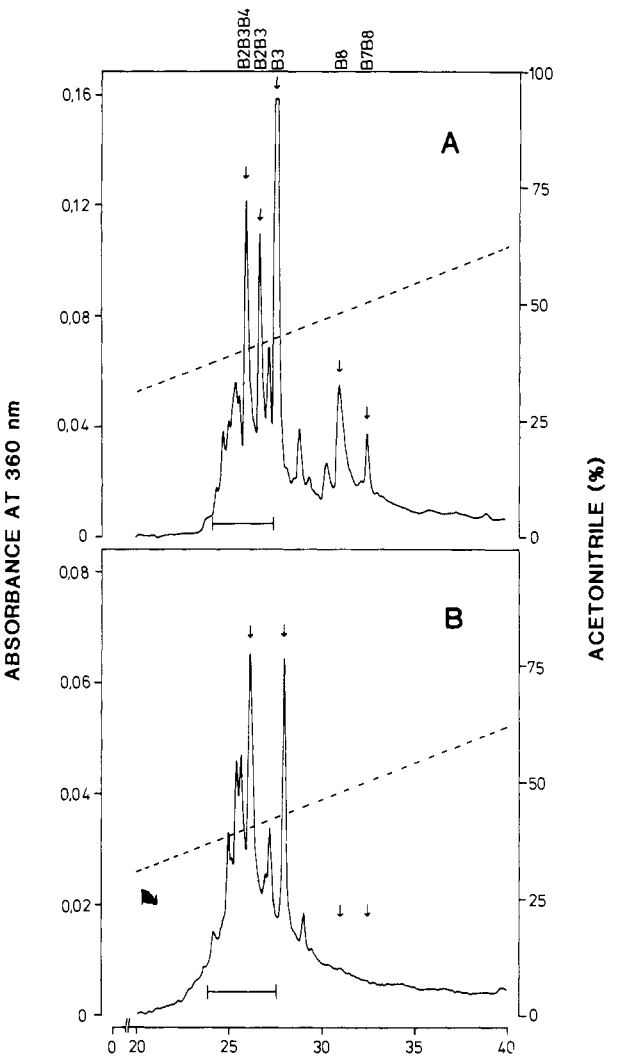

FIGURE 3: High-pressure liquid chromatography of peptides derived from CNBr-treated subunit c isolated from nitrated and subsequently reduced membrane vesicles: (A) 3-nitrotyrosine-containing peptides of nitrated subunit c; (B) 3-nitrotyrosine-containing peptides of nitrated and subsequently reduced subunit c. Right-side-out vesicles were nitrated with 15 mM tetranitromethane, and a part of the membranes was subsequently reduced with 10 mM sodium dithionite as described under Experimental Procedures. After isolation and CNBr cleavage of subunit c from those membranes, the resulting fragments were separated on a Lichrosorb RP 18 (5 μm) reverse-phase column with an acetonitrile gradient in 0.2% aqueous trifluoroacetic acid. The dashed line indicates the acetonitrile gradient used for the elution of the peptides. For the elution profile in (B), approximately half the amount of protein was applied to the column compared to that in (A). The amounts were calculated from the peak areas of B7 and B9 in HPLC chromatograms of the same samples measured at 210 nm (not shown). The eluates of the peaks designated were identified by amino acid analysis (not shown). The amounts of B3-containing larger peptides present (the position of which is marked by the bar) changed from preparation to preparation, dependent on the effectiveness of each CNBr cleavage. The observed differences are probably due to the use of hydrogen peroxide as a stopping reagent, since it can also oxidize the methionine residues of subunit c. Therefore, only the eluates of the main peaks were analyzed for their amino acid composition. The peptides were designated as described by Wachter et al. (1980).

presented in Table II), and therefore, a reduction of 3-nitrotyrosine to its amino form is only possible when the tyrosine residues of subunit c are within reach of the surrounding water phase.

After nitration and subsequent reduction of membrane vesicles with different orientation, the amount of 3-nitrotyrosine in subunit c was determined by amino acid analysis. Depending on the vesicle preparation used, different results have been obtained: in membrane vesicles with right-side-out orientation the amount of 3-nitrotyrosine reduced to 3-aminotyrosine increased with the sodium dithionite concen-

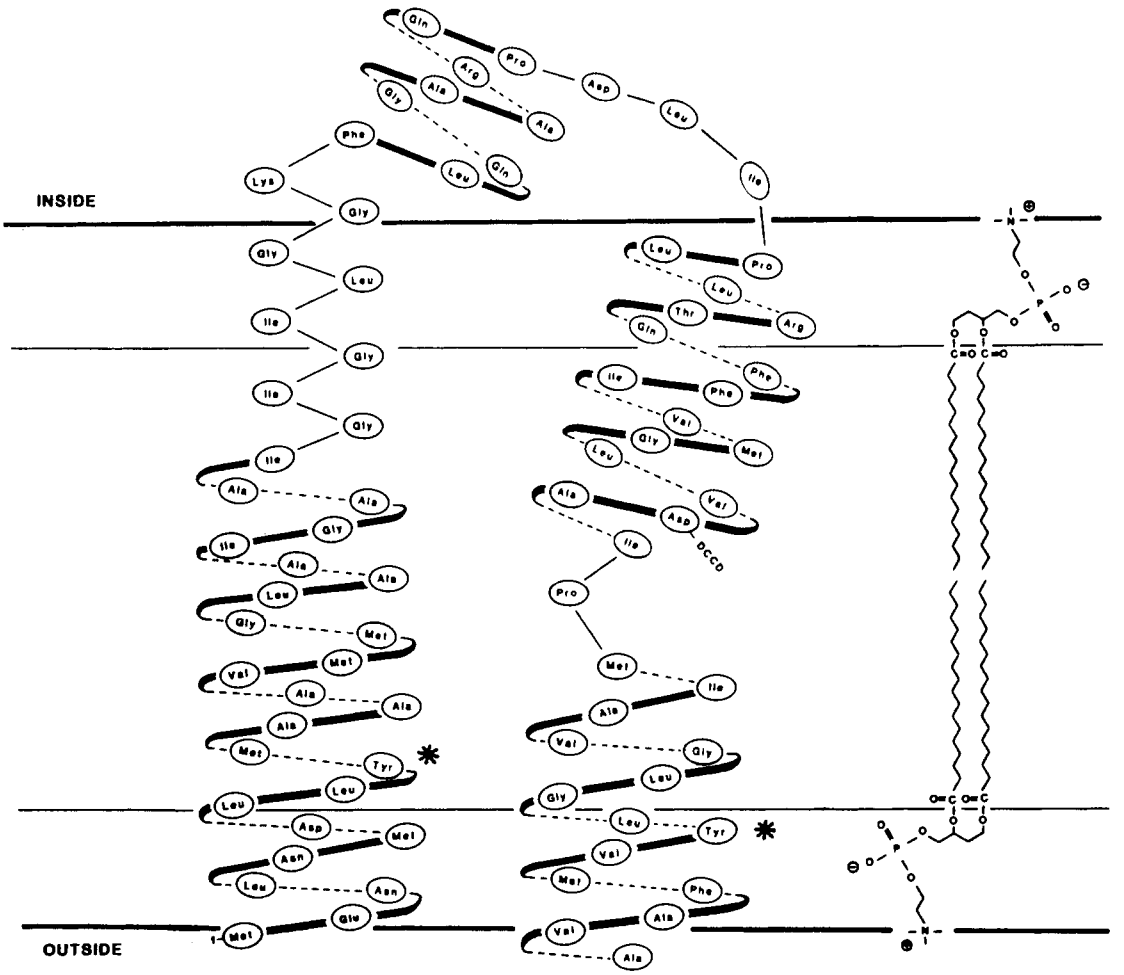

FIGURE 4: Possible arrangement of subunit c in the membrane as revealed by chemical modification (Hoppe et al., 1984; Lötscher et al., 1984), antibody binding (Loo et al., 1983; Deckers-Hebestreit & Altendorf, 1986), and amino acid substitution (Hoppe et al., 1980; Wachter et al., 1980; Fimmel et al., 1983; Mosher et al., 1985). Asterisks mark the tyrosine residues.

Table II: Reduction of 3-Nitrotyrosine Residues of Subunit c with Sodium Dithionite in Right-Side-Out and F_1-Depleted Everted Membrane Vesicles[a]

$Na_2S_2O_4$ (mM)	Tyr (%)	NO_2-Tyr (%)	NH_2-Tyr (%)
	Right Side Out		
	5.2	94.8	
5	5.2	59.3	35.5
10	5.2	35.0	59.8
	Inside Out, F_1 Depleted		
	3.4	96.6	
5	3.4	93.8	0–2.8
10	3.4	89.4	7.2

[a] The nitration and subsequent reduction of the membrane vesicles was carried out as described under Experimental Procedures. After isolation of subunit c from those membranes, the amount of tyrosine and 3-nitrotyrosine was determined by amino acid analysis.

tration applied (Table II). With 10 mM $Na_2S_2O_4$, a reduction of ~60% of the nitrotyrosine residues occurred. Interestingly, a doubling of the dithionite concentration to 20 mM produced only a 10% higher reduction, although the structure of the membrane vesicles is no longer guaranteed (data not shown). In contrast, sodium dithionite has no or only little influence on the nitrotyrosine residues in subunit c, when the incubation was carried out with F_1-depleted everted membrane vesicles (Table II).

The exclusive reduction of the 3-nitrotyrosine residues of subunit c after incubation of right-side-out vesicles with dithionite and the incomplete reduction even after incubation with high concentrations have been further investigated. After CNBr cleavage of subunit c isolated from those membranes, the tyrosine-containing peptides were separated on reverse-phase HPLC and analyzed for their 3-nitrotyrosine content. Due to the additional absorption maximum of 3-nitrotyrosine at 360 nm in acid solutions, the reduction caused by incubation with $Na_2S_2O_4$ could easily be followed during the separation of the CNBr fragments. A dithionite concentration of 10 mM resulted in a complete loss of the absorption peak (at 360 nm) of CNBr fragment B8 and also of the related peptide B7B8. In contrast, the absorption of B3 together with its related larger peptides remained nearly unaffected (Figure 3). Since different fragments containing nitrotyrosine-10 contribute to a variable extent to the overall absorption of this residue (for further explanation, see also legend to Figure 3), a quantitation is difficult to obtain. However, from data presented in Table II it can be concluded that the reduction of nitrotyrosine-10 is limited to less than 20%. With 5 mM sodium dithionite an intermediate state in reduction of peptide B8 has been observed (data not shown). Therefore, in right-side-out oriented membrane vesicles tyrosine-73 of subunit c is clearly exposed to the surrounding water phase. Tyrosine-10, however, seems to be more deeply embedded in the membrane.

Discussion

The accessibility of the tyrosine residues (Tyr-10 and Tyr-73) of subunit c in membrane vesicles has been investigated with reagents different in hydrophobicity. Tetranitromethane accumulates in the membrane due to its strong lipophilic nature and reacts preferentially from the lipid core of the membrane. The time course and the pH dependence

of the nitration showed that both tyrosine residues in subunit c were nitrated to a large extent under the conditions used. The cleavage of the nitrated protein and the separation of the resulting fragments, especially the tyrosine-containing peptides, clearly demonstrated that the distribution of the nitro group in Tyr-10 and Tyr-73 is similar at any time and at any pH value chosen. Furthermore, the percentage of nitration of each tyrosine residue in the individual peptides is always in the same range as determined for intact subunit c. These results suggest that the environments in which both tyrosine residues are located resemble each other with regard to their polar or apolar nature and to the presence of negative or positive charges.

The nitration reaction is independent of the orientation of the membrane vesicles due to the strong membrane solubility of tetranitromethane. In contrast, the reduction of nitrated residues by the ionic, water-soluble sodium dithionite requires the presence of membrane vesicles of distinct orientation. In the case of subunit c, a conversion of the 3-nitrotyrosine to the amino form only occurred when membrane vesicles with right-side-out orientation were treated with $Na_2S_2O_4$. The reduction observed was always incomplete even when high dithionite concentrations were applied. This modification convincingly locates at least one of the tyrosine residues to the periplasmic side of the membrane. On the basis of the assumption that subunit c forms a hairpin-like structure in the membrane, these results indicate that both terminal ends point to the periplasmic side of the membrane, while the hydrophilic loop is directed to the cytoplasm (see Figure 4). Such an orientation of subunit c in the membrane is also supported by analysis of an *uncE* mutant strain where the coupling between F_1 and F_o is impaired by substitution of Gln-42 in the hydrophilic region by glutamic acid (Mosher et al., 1985).

An advantage of nitration as a modification reaction is the absorption band of 3-nitrotyrosine at 360 nm in acidic solutions, which allows spectroscopic detection of the modified peptides. After reaction of the nitrated right-side-out vesicles with dithionite, no peptides containing nitrotyrosine-73 were detectable any longer, indicating a reduction to the colorless 3-aminotyrosine. In contrast, peptides containing nitrotyrosine-10 remained nearly unaffected. This result suggests that the C-terminal region of subunit c is accessible from the periplasmic side of the membrane, whereas the N terminus or at least the region around tyrosine-10 is more deeply embedded in the lipid bilayer. The accessibility from the membrane surface does not generally mean that the protein protrudes into the periplasmic space with the Tyr-73-containing region. Due to the small size of the dithionite anion, it is quite conceivable that the reagent penetrates into the region of the polar head groups of the phospholipid membrane. From this point of view, it is tempting to speculate that tyrosine-73 is directly located at the interface between the hydrocarbon "tails" and the polar head groups of the phospholipid bilayer, explaining the fact that this residue is modified by an extremely hydrophobic as well as a hydrophilic reagent.

The results obtained are in good agreement with those of other groups. Incubation of F_1-depleted everted membrane vesicles with the carbene generator TID, which is designed to label especially the hydrophobic core of membranes, resulted in a labeling of discrete amino acids at both terminal regions of subunit c also including the modification of the tyrosine residues (Hoppe et al., 1984). Incubation of membranes with the water-soluble, but at least partially permeable carbodiimide EDC, did not result in a covalent incorporation into subunit c. Instead, EDC promoted a cross-link between the C-terminal carboxyl group (Ala-79) and a near-neighbor amino group of phosphatidylethanolamine, clearly indicating that the C terminus of subunit c reaches the water phase (Lötscher et al., 1984). On the basis of these labeling data, a proper arrangement of the amino acids of the C-terminal region in the phospholipid bilayer can be deduced (Figure 4). However, from the results provided no conclusion can be drawn for the localization of the N-terminal amino acids. The TID-labeling experiments indicate that the N terminus is buried in the membrane, but the amount of radioactivity incorporated into Leu-4 and Leu-8 is very low compared to that of the other amino acids (Hoppe et al., 1984). Assuming an α-helical structure for the N-terminal region as calculated by Senior (1983) and Hoppe and Sebald (1984) and taking into account that tyrosine-10 is located in the hydrophobic core of the membrane, one can speculate that the N-terminal amino acid, which is approximately three α-helical turns apart from Tyr-10, faces the surface of the membrane. Further experiments will be necessary to obtain such a detailed picture as for the C-terminal region.

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Microcrystals of Tryptophan Synthase $\alpha_2\beta_2$ Complex from *Salmonella typhimurium* Are Catalytically Active

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ABSTRACT: An improved and efficient method has been developed for the purification of the tryptophan synthase $\alpha_2\beta_2$ complex (EC 4.2.1.20) from *Salmonella typhimurium* containing a multicopy plasmid. Microcrystals prepared in 12% poly(ethylene glycol) 8000 containing 2.5 mM spermine are shown by scanning electron microscopy to have the same crystal habit as the larger crystals that are being used for structural analysis by X-ray crystallography. The average dimensions of the crystals are 33 μm (length) × 9 μm (width) × 3 μm (maximum thickness). Our finding that suspensions of microcrystals are active in several reactions catalyzed by the active sites of the α and β_2 subunits demonstrates that both active sites are functional in the crystal and accessible to substrates. Thus the larger crystals being used for X-ray crystallographic studies should form complexes with substrates and analogues at both active sites and should yield functionally relevant structural information. A comparison of the reaction rates of suspensions of microcrystals with those of the soluble enzyme shows that the maximum rate of the crystalline enzyme is 0.8 that of the soluble enzyme in the cleavage of indole-3-glycerol phosphate (α reaction), 0.3 that of the soluble enzyme in the synthesis of L-tryptophan by the β reaction or the coupled $\alpha\beta$ reaction, and 2.7 that of the soluble enzyme in the serine deaminase reaction. These small differences in rates probably reflect functional differences between the crystalline and soluble enzymes since the reaction rates of the microcrystals are calculated to be virtually free of diffusional limitation under these reaction conditions. The observed larger differences in the effects of ligands on the kinetic constants suggest that the transmission of ligand-induced conformational changes from one subunit to the other is reduced in the crystal due to crystal lattice forces.

The purpose of this work is to compare the functional properties of the crystalline $\alpha_2\beta_2$ complex of tryptophan synthase (EC 4.2.1.20) from *Salmonella typhimurium* with the properties of the enzyme in solution. The results are important in determining whether the structure of the enzyme which is being determined by X-ray crystallography (Ahmed et al., 1985)[1] is that of an active form of the enzyme, whether the crystalline enzyme can bind substrates and analogues, and whether the crystalline enzyme can undergo the same lig-

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[1] A structural determination is in progress (C. C. Hyde, E. A. Padlan, S. A. Ahmed, E. W. Miles, and D. R. Davies). A preliminary report will be presented at the meeting of the American Society of Biological Chemists in Philadelphia, PA, June 8-11, 1987 (Hyde et al., 1987).