The γ Subunit of the Escherichia coli F1-ATPase Can Be Cross-linked Near the Glycine-rich Loop Region of a β Subunit When ADP + Mg2+ Occupies Catalytic Sites But Not When ATP + Mg2+ Is Bound*

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A mutant of the Escherichia coli F1-ATPase, γS8C, has been reacted with a novel bifunctional reagent, N-maleimido-N′-(4-azido-2,3,5,6-tetrafluorobenzenamido)cystamine (TFPAM-SS1). Modification of Cys-8 via the maleimide, followed by photolysis to convert the azido group to a reactive nitrene, led to cross-linking of the γ subunit to a β subunit. When this cross-linking was conducted with ADP + Mg2+ in catalytic sites, the predominant cross-linked product had a Mr of 108,000. If cross-linking was done with uncleaved ATP + Mg2+ in catalytic sites, cross-linked products of 102,000 and 84,000 were formed. Cross-linking under both conditions led to inhibition of ATPase activity. TFPAM-SS1 could be cleaved by using reducing agents to break the disulfide bond that links the maleimide and tetrafluorophenylazide moieties. Cleavage of this disulfide bond after formation of 102,000 and 84,000 species led to full recovery of ATPase activity. When the 108-kDa cross-linked product was cleaved, full activity was not restored, presumably because of insertion of the tetrafluorophenylazide into a functionally important site on the β subunit. After cleavage of the disulfide bond, the free thiol could be reacted with [14C]N-ethylmaleimide, thereby radioactively tagging the sites of insertion of the tetrafluorophenyl nitrene moiety. In this way, the site of cross-linking from Cys-8 of γ to the β subunit in the presence of ADP + Mg2+ was localized within the sequence Val145-Lys155, which contains the glycine-rich loop. This loop region is a part of the catalytic site of the enzyme.

Energy transducing membranes, including the plasma membrane of bacteria, the thylakoid membrane of chloroplasts, and mitochondrial inner membrane all contain a structurally homologous F1,F0-ATP synthase which uses the energy of a transmembrane proton gradient generated by oxidative or photophosphorylation to synthesize ATP from ADP and inorganic phosphate.

The ATP synthase is organized into two distinct domains. Extrinsic to the membrane bilayer is an F0 part comprised of five subunits, α, β, γ, δ, and ε, in the stoichiometry 3:3:1:1:1, which contains the catalytic sites located mainly on β subunits. Spanning the membrane bilayer is the F0 part, composed of three subunits, a, b, and c, in the stoichiometry 1:2:10–12, which is involved in the proton-channeling function of the enzyme complex (reviewed in Senior, 1988; Futai et al., 1989; Cross, 1988).

Our structural studies on the Escherichia coli enzyme indicate that the F1 part is roughly globular with the α and β subunits arranged hexagonally and alternating, i.e. α-β-α around a central cavity (Gogol et al., 1989a, 1989b). These major subunits are interdigitated for most of their length to give a barrel-like structure. The γ subunit is located mainly within the cavity as indicated by cryoelectron microscopy experiments (Gogol et al., 1989b; see also Ishii et al., 1993) but is exposed at one end based on antibody binding (Aggeler et al., 1990) and protease digestion studies (Gavilanes-Ruiz et al., 1988; Bragg and Hou, 1987; Mendel-Hartvig and Capaldi, 1991a). The δ and ε subunits are bound more peripherally to the α3β3γ core of the enzyme complex (Gogol et al., 1989b), with the ε subunit interacting with both the γ and β subunit (Aggeler et al., 1992; Lätscher et al., 1984; Dallmann et al., 1992).

To define structural features more precisely, and to examine conformational changes in ECF,F01 that are involved in coupling catalytic site events and the proton pumping function, we have created mutants of the enzyme in which Cys residues are incorporated in individual subunits. These introduced Cys residues can then be used to react with reporter groups such as spin labels, fluorophores, heavy atom clusters, and cross-linking reagents (Wilkens and Capaldi, 1992; Aggeler et al., 1992; Aggeler and Capaldi, 1992). For example, we recently produced five mutants of the γ subunit, S8C, S81C, T106C, S179C, and V286C and used these in cross-linking experiments, employing the novel bifunctional reagents TFPAMs (Aggeler and Capaldi, 1992). These reagents contain a maleimide at one end which can be reacted with the introduced Cys, and they have a tetrafluorophenylazide as the other reactive group (Keana and Cai, 1989) which is photoactivatable to a nitrene that can insert into near neighbor regions of a protein complex with high efficiency. Using this cross-linking approach, the N terminus of γ (specifically, the Cys at position 8) was found to react with a β subunit, while the C terminus (the Cys at position 286) was cross-linked to an α subunit (Aggeler and Capaldi, 1992).

Cross-linking from Cys-8 of the γ subunit to the β subunit proved to be sensitive to the nucleotide occupancy of catalytic sites. With ATP + Mg2+ bound, the major cross-linked prod-

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§The abbreviations used are: ECF, Escherichia coli F1-ATPase; AMP-PNP, 5′-adenylyl-β,γ-imidodiphosphate; DTT, dithiothreitol; HPLC, high pressure liquid chromatography; MOPS, 3-[N-morpholino]propanesulfonic acid; NEM, N-ethylmaleimide; TCEP, Tris-(2-carboxyethyl)phosphine; TEAP, triethylamine/phosphoric acid; TFPAM, tetrafluorophenylazidemaleimide; rpm, counts/min.

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ucts formed had $M_r \sim 102,000$ and 84,000. When ADP + Mg$^{2\+}$ were present, the predominant cross-linked product had a molecular mass of approximately 108,000; the 102-kDa product was present by small amounts, while the 84-kDa species was absent (Aggerel and Capaldi, 1993).

Here we describe cross-linking experiments using the prototype of a new generation of TFPAMs in which the two functional ends are bridged by a disulfide bond, making the covalently cross-linked products cleavable by reducing agents. With this reagent we have reproduced the nucleotide-dependent cross-linking from Cys-8 of the $\gamma$ subunit to a $\beta$ subunit, and have been able to map the site of insertion under conditions of ADP + Mg$^{2\+}$ to an 11-residue peptide of the $\beta$ subunit that includes the glycine-rich loop which is part of the catalytic site.

**EXPERIMENTAL PROCEDURES**

*N-Maleimidocystamine*—A solution of 11 g of cystamine dihydrochloride (compound 1 in Fig. 1) in 100 ml of water was treated with 13 ml of 20% aqueous NaClO, and the resulting mixture was extracted with CHC1$_3$ ($3 \times 50$ ml). The CHC1$_3$ extract was evaporated to leave 2.3 g of compound 2 as a liquid: $^1$H NMR (CDCl$_3$) $\delta$ 1.376 (bs, 4), 2.757 (t, J = 6.0 Hz), 3.015 (s, 4). The sample was dissolved in dry THF (40 ml), and a solution of 1.5 g of maleic anhydride in dry THF (20 ml) was added dropwise to this mixture precipitously, and stirring was continued for 1 h. The mixture was filtered and the resulting white solid compound 3 was washed with ether ($2 \times 30$ ml) and dried. A solution of the solid in dimethyl sulfoxide (12 ml) and trifluoroacetic acid (6 ml) was heated at 90°C for 20 h. The solution was then added dropwise to ether (200 ml) to produce an oily precipitate. The ether was decanted leaving a pale orange oil which was flash chromatography over silica gel (Merck, Darmstadt, West Germany) with CHC1$_3$-$\text{EtOH}$-concentrated HCl giving several fractions showing a single spot at $R_f = 0.3$ on thin layer chromatography (same solvent system). These were combined and concentrated to dryness giving 2.7 g (52%) of crude maleimide 4 as an oily salt: $^1$H NMR (CDCl$_3$) $\delta$ 2.881 (t, J = 6.6 Hz, 2), 3.041 (t, J = 6.6 Hz, 2), 3.310 (m, 2), 3.528 (t, J = 6.6 Hz, 2), 6.716 (s, 2). High resolution mass spectroscopy was calculated for C$_{13}$H$_{18}$NO$_6$S$_2$, M$^+$ 233.0418. Found: 233.0421.

*N-Maleimido-N'-(4-azido-2,3,5,6-tetrafluorobenzenamido)-cystamine 8 (TFPAM-SS1)*—To a stirred solution of 367 mg (1.1 mmol) of crude 4 in CHC1$_3$ (10 ml) was added 100 Molecular Sieve beads (8-12 mesh, Type 3A, Grade 546, Davison) as a dehydrating agent. After 1 h at 25°C, the mixture was treated with 550 mg (5.4 mmol) of triethylamine and then it was allowed to stir at 25°C for 30 min. To the mixture was added 314 mg (0.56 mmol) of N-succinimidyl 4-azido-2,3,5,6-tetrafluorobenzenesulfonate (Pierce Chemical Co., Rockford, IL) in CHC1$_3$ ($2 \times 50$ ml). The mixture was stirred for 1 h and then it was filtered. The filtrate was purified in portions by preparative thin layer chromatography over silica gel (Merck Art. 7477). Kieselgel 60 PF$_{254}$; developing solvent: ethanol, 10:1; CHC1$_3$-hexane-MeOH). The $R_f$ 0.55 band was eluted with 10:1 CHC1$_3$-MeOH to give 171 mg (58%) of 8 as a colorless solid. The compound was further purified by treatment with acetone (6 ml), filtration, and then dropwise addition of the filtrate into hexane (28 ml). The precipitate was obtained by centrifugation and dried to yield 135 mg of 8 as a colorless solid, mp 118-120°C (decomp): $^1$H NMR (CDCl$_3$) $\delta$ 2.882 (t, J = 6.9 Hz, 2), 2.978 (t, J = 6.0 Hz, 2), 3.780 (m, 1), 5.614 (m, 1), 6.656 (m, 1), 7.128 (m, 1), 7.589 (m, 1, 2), 8.167 (m, 1). High resolution mass spectroscopy was calculated for C$_{19}$H$_{16}$F$_{15}$N$_4$O$_5$S$_2$, M$^+$ 763.9178. Found: 763.9158.

**RESULTS**

This work introduces a new cross-linker, a variant of the TFPAMs used earlier (Aggerel et al., 1992) that has, additionally, a disulfide bond in the linker region between the maleimide and tetrafluorophenylazole moieties (Fig. 1). The distance between functional groups is 17.5 Å. TFPAM-SS1 was used in cross-linking studies of the ECF, mutant $\gamma$S8C. We have shown that TFPAM-6 cross-links from the introduced Cys residue at position 8 on the $\gamma$ subunit to a $\beta$ subunit and that this cross-linking is sensitive to nucleotide binding in catalytic sites (Aggerel and Capaldi, 1993). As shown in Fig. 1. Synthesis of the cleavable heterofunctional photo-cross-linker TFPAM-SS1.

![Fig. 1. Synthesis of the cleavable heterofunctional photo-cross-linker TFPAM-SS1.](image-url)
Fig. 2A, the γ subunit is also cross-linked to a β subunit by TFPAM-SS1 in a nucleotide-dependent manner. Thus, when photolyzed in the presence of ATP + EDTA (Fig. 2, lane 3), AMP · PNP + Mg²⁺ or with ATP + Mg²⁺ added along with the inhibitor azide (results not shown), two major cross-linked products were generated with a molecular mass of ~102 and ~84 kDa. These are the same sized products observed using TFPAM-6. When cross-linking was conducted in the presence of ADP + Mg²⁺, AMP · PNP + Mg²⁺ or when ADP + Pₗ + Mg²⁺ were generated in catalytic sites by adding ATP + Mg²⁺ and allowing enzyme turnover (Fig. 2, lane 4), the predominant cross-link product had a molecular mass of 108 kDa; again, the same sized product as with TFPAM-6.

The major advantage of TFPAM-SS1 over TFPAM-6 is that the disulfide bond in the spacer can be broken to cleave the cross-link. This has allowed us to evaluate the relative importance for the activity of ECF, of covalent cross-linking of the two subunits (γ to β) as opposed to chemical modification by the functional ends. The availability of a cleavable bifunctional reagent that generates cross-linked products in high yield also allows identification of the site(s) of insertion into the protein complex, as discussed later.

Several reducing agents were examined for their effectiveness in breaking the disulfide of TFPAM-SS1. Tris-(2-carboxyethyl)phosphine (TCEP) was chosen for cleaving this bond when subsequent[^14C]NEM labeling was being conducted because it was effective at low concentrations. DTT was used when activity measurements were being done. The activity effects of cross-linking from the Cys at position 8 of the γ subunit with TFPAM-SS1 are reported in Table I. Reaction of ECF, isolated from the mutant γS8C with this reagent had only a small effect on ATPase activity without photolysis to generate cross-linking whether ATP + EDTA or ADP + Mg²⁺ + Pₗ were present. When photolysis was carried out in EDTA + ATP, the inhibition was 34 and 44% in two separate experiments. In ATP + Mg²⁺ (Pₗ + ADP + Mg²⁺), the inhibition was 36 and 42% in two sets of experiments. These levels of inhibition were proportional to the yield of cross-linked products in the different samples based on the disappearance of the γ subunit in Coomassie Blue-stained polyacrylamide gels.

Subsequent reduction of the disulfide bond restored essentially full activity to the sample cross-linked in ATP + EDTA. However, cleavage of the disulfide bond in samples cross-linked in ATP + Mg²⁺, and containing the 108-kDa species, failed to restore full activity. In the data set in Table I, the inhibition was 20% even after complete cleavage of the cross-linked products (Fig. 2B). This implies that insertion of the tetrafluorophenylethylene moiety into the β subunit has modified a site important for ATPase activity.

Using TFPAM-SS1 provides a means of tagging the site of insertion into the β subunit for subsequent peptide mapping. In this approach the disulfide bond generated by cross-linking is broken with the phosphine (see Fig. 2C) and then the free thiols are reacted with[^14C]NEM. Radioactive NEM will be incorporated into the γ subunit via the SH provided by the cross-linker attached at position 8, and into the β subunit via the SH incorporated as part of the tetrafluorophenylazide moiety (Fig. 3). The few intrinsic Cys residues in the ECF complex, i.e., Cys-140 of the β subunit and an as yet unidentified Cys in one of the three α subunits, will also be modified (Mendel-Hartvig and Capaldi, 1991b) but do not affect the analysis.

The only intrinsic Cys in the β subunit (Cys-137) is buried in the native protein and, thus, is not available for modification by maleimides. Therefore, location of the radioactivity by peptide mapping and sequencing identifies only the site(s) of insertion of the cross-linker into the β subunit. In two different experiments, 0.12 mol of[^14C]NEM were incorporated into the β subunit after cross-linking in ATP + Mg²⁺ followed by disulfide bond reduction and then reaction with the maleimide. This is equivalent to a yield of cross-linking of 36% from Cys-8 to β subunit(s), consistent with the quantitation from Coomassie Blue-stained gels (see Aggeler and Capaldi, 1992).

To localize the site of modification of the β subunit, samples of ECF, that had been cross-linked under different nucleotide conditions were reacted with TCEP and then with[^14C]NEM, subunits were separated by SDS-polyacrylamide gel electro-

### Table I

<table>
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<td>12.4</td>
<td>12.0</td>
<td>11.2</td>
</tr>
</tbody>
</table>

*ATPase was kept for 2 h in the dark after maleimide modification and removal of excess label.
^ATPase was photolyzed for 2 h with a 6-watt 365 nm UV lamp. Photolysis of unmodified ATPase for 3 h led to a loss of less than 5% of the activity in the presence of ATP + EDTA or ATP + Mg²⁺.
^ATPase was reacted with 50 mM DTT for 1 h after cross-linking.

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Fig. 2. Photo-cross-linking of γS8C ATPase with cleavable cross-linker TFPAM-SS1. 1 mg of ATPase was depleted of nucleotides and labeled in 100 μl of 50 mM MOPS, pH 7.0, 0.5 mM EDTA, and 10% glycerol by adding 1 μl of 20 mM TFPAM-SS1 (in dimethyl sulfoxide) and incubating for 1 h at room temperature. After removal of excess label, 5 mM ATP (lanes 1 and 3) or 5 mM ATP + 5.5 mM MgCl₂ (lanes 2 and 4) were added. Samples were either kept in the dark (lanes 1 and 2) or photolyzed with a 6-watt 365 nm UV lamp (lanes 3 and 4) for 2 h at room temperature. A, 150 mM NEM was added to each sample of 200 μg of ECF, and incubated for 1 h before addition of dissociation buffer without reducing agent and application on a SDS-containing 10–18% polyacrylamide gel. B, samples identical to A were incubated with 50 mM DTT for 3 h before the addition of 150 mM NEM. C, samples containing 170 μg of ECF, were reduced by incubation for 5 h with 1.5 mM TCEP after adding to the Mg-free samples (lanes 1 and 3) 5.5 mM MgCl₂. 50 mM iodoacetamide was added for 1 h followed by dissociation buffer without reducing agent and application on a gel.
FIG. 3. Identification of cross-link products on amino acid level. Polypeptide 1 containing a cysteine group (γ subunit of ECF₁) is reacted with the maleimido group of TFPAM-SS₁. Polypeptide 2 (β subunit) is then modified by photolysis via a nitrene insertion. After reduction with Tris-(2-carboxyethyl)phosphine, thiol groups are reacted with [¹⁴C]NEM. Subunits are separated by SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, cleaved by protease (trypsin), and the fragments separated on reverse-phase HPLC and analyzed by N-terminal peptide sequencing.

FIG. 4. Fractionation of tryptic fragments of ECF₁ β-subunit by HPLC. 2 mg of ECF₁ (5 nmol) was cross-linked with TFPAM-SS₁, carrying out the photolysis in the presence of Mg²⁺, ADP + P₃. Cross-linked products were cleaved with 1.5 mM TCEP for 5 h. After passage through a centrifuge column in 50 mM MOPS, pH 7.0, 0.5 mM EDTA and 10% glycerol, [¹⁴C]NEM was added to a final concentration of 200 μM for a 90-min incubation. The reaction was quenched with 20 mM cysteine for 30 min, and after addition of 100 mM NEM and dissociation buffer without reducing agent, the sample (1.2 ml) was applied on a 15-cm wide lane of a SDS-containing 10% polyacrylamide gel (20 cm long). The β subunit was transferred onto a nitrocellulose membrane and cleaved with trypsin overnight at 37 °C. The peptide fragments were recovered in 2 ml containing 5000 cpm. A 0.35-ml sample was applied on a C₁₈ reverse-phase HPLC column after adjusting the pH to 7 by addition of 2.5 μl of 25% phosphoric acid. Peptide fragments were eluted in the triethylammonium/phosphoric acid system described under “Experimental Procedures” with a 0-70% acetonitrile gradient. The histogram shows the radioactivity distribution and the solid line absorption at 220 nm. The arrows indicate the position of unmodified (a) or modified VGLFGGAGVGK (b).

Peroxidation, and the β subunit blotted onto nitrocellulose and cleaved by trypsin in a buffer containing 5-10% CH₃CN while still attached to the solid support. Fragments were separated by HPLC. Fig. 4 shows a typical profile of tryptic fragments of the β subunit in HPLC using a gradient from 0 to 70% CH₃CN in TEAP buffer. The sample analyzed in Fig. 4 had been cross-linked in ATP + Mg²⁺ and the predominant cross-linked product was the 108-kDa species. In all, 55% of the total radioactivity eluting from the column ran in a small peak at 109 min in the peptide profile. No other peak contained more than 10% of the total radioactivity. The small size of the [¹⁴C]NEM-containing peak is expected, given that a yield of cross-linking of around 30-40% leads to modification and radioactive labeling of only 10-13% of the β subunit, and hence, at most 10-13% of any fragment of the polypeptide.

Fig. 5 compares the HPLC profiles of tryptic fragments obtained after cross-linking in ATP + EDTA (i), in ATP + Mg²⁺ (iii), and includes a dark control for the ATP + Mg²⁺ conditions. It can be seen that the small peak at 109 min (Fig. 5B), and radioactivity eluting from the column (Fig. 5A) at this position, are found only when the 108-kDa species is generated, not when the 84 or 102-kDa species are predominant, and not in the dark control. With samples cross-linked in ATP + EDTA, radioactivity was distributed more broadly, there was no single peak in the HPLC profile that contained a majority of the [¹⁴C]NEM, indicating either several sites of modification by the tetrafluorophenylazide and/or instability of the products of the 102- and 84-kDa cross-linked species during sample preparation for HPLC, preventing further analysis. Some of these multiple sites of labeling are the background labeling in ATP + Mg²⁺, as there is some cross-linking into the 102-kDa species.

To locate the site of insertion of the cross-linker into the β subunit in the 108-kDa species more precisely, the [¹⁴C]NEM-labeled tryptic fragment was subjected to N-terminal protein sequencing. Four sequences were identified, the predominant one beginning with VGLF, the minor ones having the N-terminal sequences TGSI, NIAI, and LVLE. In two different experiments the sequence VGLF was found at a ratio of between 4:1 and 5:1 with respect to the other, minor, sequences identified. Sequencing was conducted for several other peaks from the HPLC, and a major peak containing the sequence VGLF was found to elute at 70 min (Fig. 4a).

The presence of most of the fragment VGLF eluting at 70 min, with only a minor peak of this fragment eluting at 109 min, is evidence that the latter is modified to make it more hydrophobic, as would be expected from incorporation of the tetrafluorophenylazide moiety. The molar concentration of the modified VGLF based on the yield of the amino acids in the first three cycles was consistent with the molar concen-
tration of \[^{14}C\]NEM in the peak, while the yields of TGSI and other minor sequences were four to five times lower than the estimated molar concentration of NEM.

The above results strongly suggest that the \(\gamma\) subunit is cross-linked from the Cys at position 8 to the \(\beta\) subunit by insertion into the sequence \[^{14}C\]VGLFGGAGVGK\[^{35}\].

More definitive evidence for this cross-linking site was obtained by using two HPLC separation steps to resolve fragments, as shown in Fig. 6. In this approach, tryptic fragments were first separated with a gradient of CH\(_3\)CN (10-70\%) in trifluoroacetic acid buffer, which resolved a peak at 85 min containing 60% of the radioactivity. This peak was then chromatographed on a column in 0-70\% CH\(_3\)CN in TEAP buffer. The peak at 109 min contained >90% of the radioactivity from 85-119 min is shown in the histogram (A), and the absorption at 220 nm from 85-119 min is shown in B. The arrows indicate the position of modified VGLFGGAGVGK.

**DISCUSSION**

Here we introduce a new cross-linker, TFPAM-SS1, which has a maleimide and a tetrafluorophenylazide as functional groups separated by a linker which includes a disulfide bond. The reagent can be reacted with cysteine residues, either endogenous to the protein being studied, or introduced at sites of interest by site-directed mutagenesis as is the case in our study of ECF, reported here. Photolysis of the protein-TFPAM-SS1 adduct induces covalent cross-linking by converting the tetrafluorophenylazide to a nitrene, and as we have shown before, the yield of cross-linking is generally high with tetrafluorophenylazides because the presence of fluorines in the phenyl ring prevents internal rearrangement reactions (Keana and Cai, 1990; Aggeler et al., 1992). After cross-linking, any effects on ATPase activity can be analyzed for the role of covalent linkage of two sites versus the chemical modification of single sites, either for the maleimide which can be assessed before photolysis, or by insertion of the tetrafluorophenylnitrene which can be assessed after breaking the disulfide bond.

We have used TFPAM-SS1 to examine the cross-linking between Cys introduced at position Ser-8 in the \(\gamma\) subunit and the \(\beta\) subunit in ECF, under different nucleotide conditions. Studies with TFPAM-SS1 confirm our previous experiments with TFPAMs in showing that the cross-linked products formed between the Cys in \(\gamma\) to the \(\beta\) subunit are nucleotide-dependent (Aggeler and Capaldi, 1992, 1993). In ADP + Mg\(^{2+}\), whether Pi was present or not, the major cross-linked product had a \(M_r\) of 108,000. By cleaving the cross-linked product, labeling with \[^{14}C\]NEM, and then subsequently peptide mapping of the \(\beta\) subunit, we were able to locate the predominant site of cross-linking from Cys-8 of \(\gamma\) in ADP + Mg\(^{2+}\), i.e. before the ATP has been hydrolyzed to ADP, or with AMP-PNP +
**E. coli F$_1$-ATPase β-γ Subunit Proximity**

**FIG. 6.** Two-step purification of the major radioactive peptide fragment of trypticized ECF, β subunit by HPLC. 2 mg of ECF, was cross-linked with TFPAM-SS1 (photolysis in Mg$^{2+}$, ADP + Pi) and the β subunit isolated and trypsinized as described in Fig. 4. 12,000 cpm could be recovered in a total of 2.5 ml. A, to a sample of 0.5 ml, 19 µl of 10% trifluoroacetic acid was added before injection on a C$_18$ reverse-phase HPLC column. A 10–70% acetonitrile gradient in the trifluoroacetic acid system (“Experimental Procedures”) was used for separation of the tryptic fragments. The regions indicated by bars contained 100 cpm (a), 210 cpm (b), 730 cpm (c), and 100 cpm (d). Fractions from 83–88 min (region c) containing 60% of the recovered radioactivity were pooled from four column runs yielding 2400 cpm. B, the pooled fractions from A (peak c) were concentrated with a SpeedVac at room temperature and applied on the same HPLC column in the triethylamine/phosphoric acid system (0–70% acetonitrile). The majority of the radioactivity (2,080 cpm) was recovered in a peak at 109 min.

**FIG. 7.** Peptide sequence analysis of major radioactive tryptic fragment of ECF, β subunit. The pooled fractions at 109 min from three HPLC column runs (450 pl each) described in Fig. 4 were concentrated with a SpeedVac. The 2960 cpm obtained represent around 80 pmol of peptide (7 nCi/mmol [3H]NEM is 12 cpm/pmol). 65 pmol of the peptide was applied on a protein sequenator.

Mg$^{2+}$ in catalytic sites, the 108-kDa product is only a minor species and the predominant cross-linked products obtained have molecular masses of ~102 and ~84 kDa. These same two species are also produced when cross-linking is conducted in either ADP or ATP without Mg$^{2+}$ present, i.e. in the presence of EDTA (Aggeler and Capaldi, 1993).

Our previous studies have established that switching between the 108-kDa species in ADP + Mg$^{2+}$ and the 102- and 84-kDa species in ATP + Mg$^{2+}$ depends on the presence of the ε subunit (Aggeler and Capaldi, 1993). When the ε subunit is missing, only the 102- and 84-kDa species are formed, even in ADP + Mg$^{2+}$. As the enzyme is a highly active ATPase without the ε bound (Tuttas-Dorschug and Hanstein, 1989; Aggeler et al., 1990), the structural alterations being monitored by changes in cross-linking cannot be a necessary part of the conversion of ATP to ADP. Instead, we believe that the structural changes represent conformational changes in the γ subunit that are a part of the coupling of catalytic site events with proton translocation through the F$_0$. Further, we conclude that binding of the ε subunit by γ and β subunits is required to maintain the γ subunit in a form in which it can sense and respond to catalytic site events.

The emerging model of F$_1$-ATPase is one in which the three copies of each of the α and β subunits form a hexagon surrounding a central cavity of ~30 Å into which the γ subunit extends for at least 35 Å and possibly the full 90 Å of the height (Gogol et al., 1989a, 1989b; Abrahams et al., 1993; Ishii et al., 1993). Our observations that the γ subunit can be cross-linked to the peptide fragment 145–155 of the subunit by our observations that the γ subunit can be cross-linked to the peptide fragment 145–155 of the subunit has important structural implications. This peptide contains the so-called glycine-rich loop (residues 149–155) (Walker et al., 1984) which is now unequivocally established to be a part of the catalytic site (Duncan and Cross, 1992; Futai et al., 1992).

Our results, therefore, place the catalytic sites in the interior part of the F$_1$ structure rather than at the periphery of the 90-Å diameter complex. This is consistent with previously difficult-to-interpret fluorescence energy transfer measurements which had placed the catalytic sites within 40–45 Å of each other (Snyder and Hammes, 1984; Cerione and Hammes, 1982).

Recent evidence indicates that the catalytic sites of F$_1$ are constructed similarly to the GTP-binding site of the Ras protein (Duncan and Cross, 1992; Futai et al., 1992). A high resolution structure of Ras is available and information on the structural changes occurring when GTP is hydrolyzed to GDP has been obtained (Milburn et al., 1990; Wittinghofer and Pai, 1991). In Ras, and therefore by analogy in F$_1$, the glycine-rich loop participates in binding the β and γ phosphate groups of ATP and in liganding the Mg$^{2+}$ ion. Movements of this sequence and rearrangements of other close-by segments of the Ras protein occur on GTP hydrolysis, and these changes alter the binding of other proteins with Ras. Similar changes in and around the catalytic site of F$_1$ could trigger changes in the structure of the γ subunit under coupling conditions, which are then transduced to the F$_0$ to drive proton translocation across the plasma membrane in the case of the bacterial enzyme, and the mitochondrial inner membrane in the case of mitochondrial F$_1$.

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