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The *Escherichia coli* F_1-ATPase mutant βTyr-297 → Cys: functional studies and asymmetry of the enzyme under various nucleotide conditions based on reaction of the introduced Cys with *N*-ethylmaleimide and 7-chloro-4-nitrobenzofurazan

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Abstract

Conversion of residue βTyr-297 of the *Escherichia coli* F_1-ATPase (ECF_1) to a Cys in the mutant βY297C led to impaired oxidative phosphorylation based on growth curves. The ATPase activity of ECF_1 isolated from the mutant βY297C was only 1% of wild-type activity, but the residual activity involves cooperative multi-site enzyme turnover based on inhibition by DCCD and azide. ATPase activity could be increased to 8%, and 13% of wild-type by reaction of the introduced Cys with *N*-ethyl maleimide (NEM), and 7-chloro-4-nitrobenzofurazan (NbfCl), respectively, suggesting that enzymatic function is improved by an increased hydrophobicity of residue βCys-297. The mutation βTyr-297 → Cys had no effect on nucleotide binding in studies with the fluorescent analog *lin*-benzo-ADP. The asymmetry of ECF_1 was investigated in the mutants βY297C and βY297C:E381C/εS108C by examining the relative reactivity of Cys-297 in the three copies of the β subunit under different nucleotide binding conditions. In agreement with a previous study (Haughton, M.A. and Capaldi, R.A. (1995) J. Biol. Chem. 270, 20568–20574), the asymmetry was maintained under all nucleotide conditions. The NbfCl reaction site was found to be β_{free}, which is also the site most reactive to NEM, β_ϵ is the second site which reacts with NbfCl or NEM, while the third site, β_γ, is poorly reactive to either reagent.

Keywords: ATPase, F_1-; Asymmetry; Chemical labeling; NbfCl

1. Introduction

The H^+-translocating F_1F_0-ATPase (ATP synthase) directs the synthesis of ATP from ADP and inorganic phosphate (P_i) in bacteria, mitochondria, and chloroplasts during oxidative- or photo-phosphorylation [1–4]. In the reverse direction, the enzyme can also generate an ATP hydrolysis-driven proton gradient for use in ion transport processes. The F_1F_0 from *Escherichia coli* (ECF_1F_0) is a multi-subunit complex of molecular weight 530 000 Da. The F_1 part is composed of five subunits (α, β, γ, δ, and ε, in the stoichiometry 3:3:1:1:1), while the F_0 part is composed of three different subunits (a, b, and c, in the ratio 1:2:10–12). They are linked by a stalk region comprised of subunits from both F_1 (γ, δ, and ε) and F_0 (b) [4–7].

Earlier electron microscopy studies of ECF_1 [8,9], and the recent X-ray structure determination of MF_1 from bovine heart mitochondria [10], show that the α and β subunits alternate in a hexagonal arrangement around a central cavity that encloses a part of the γ subunit. The catalytic sites are located primarily on the β subunits. Additional nucleotide binding sites are present on the α subunits that may play a structural rather than a catalytic role. The crystal form of the enzyme reported by Abrahams et al. [10] shows an asymmetry of the three α-β subunit pairs that is reflected both in different nucleotide

Abbreviations: ECF_1, soluble portion of the *Escherichia coli* F_1F_0, ATP synthase; ECF_1F_0, *Escherichia coli* F_1F_0 ATP synthase; CF_1, soluble portion of the chloroplast F_1F_0-ATPase; MF_1, soluble portion of the mitochondrial F_1F_0-ATPase; TF_1, soluble portion of the thermophilic bacterium PS3 F_1F_0-ATPase; CM, *N*-[4-[7-(diethylamino)-4-methyl]coumarin-3-yl] maleimide; Mops, 3-(*N*-morpholino)propanesulfonic acid; NEM, *N*-ethylmaleimide; DCCD, dicyclohexyl carbodiimide; NbfCl, 7-chloro-4-nitrobenzofurazan; AMP·PNP, 5′-adenylyl-β,γ-imidodiphosphate; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; Me_2SO, dimethyl sulfoxide; PAGE, polyacrylamide gel electrophoresis.

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occupancies of the catalytic sites and different interactions of each α-β pair with the γ subunit. ATP synthesis and hydrolysis are highly cooperative processes involving three catalytic sites [4,11]. Evidence from genetic [12–14], biochemical [15–18], and structural studies [10,19] supports a key role for the γ subunit in both catalytic cooperativity and energy coupling.

A number of nucleotide analogs have been used to explore the properties of the catalytic sites in F_1-ATPases, including 8-azido ATP and NbfCl, both of which have been shown to modify βTyr-297 of ECF_1 or the homologous residues of MF_1, TF_1 or CF_1 (βTyr-311, βTyr-307, or βTyr-328 respectively), and inhibit enzyme activity essentially fully [20–25]. In apparent contradiction, Odaka et al. [26] have reported that conversion of Tyr-307 (the equivalent of Tyr-297) to a Cys in TF_1 has no effect on activity. To learn more about any possible functions of Tyr-297, we have mutated this residue to a Cys in ECF_1. Incorporation of a Cys in the β subunit also allows us to study the asymmetry of the enzyme by examining the relative reactivity of this residue in the different β subunits of ECF_1 under different nucleotide conditions.

2. Materials and methods

2.1. Strains and construction of plasmids containing mutations in the uncD and uncC genes

A 1.01-kb *Nco*I fragment encoding the COOH-terminal part of the β subunit was isolated from the *unc*-operon containing plasmid pRA100 and inserted into M13mp18 (New England Biolabs) for the site-directed mutagenesis step as described in Aggeler et al. [27]. The oligonucleotide CATCCGCAGGTACG*C*ATACTGCCTGTAC was used to replace a tyrosine with a cysteine at position β297 [28]. Incorporation of the mutation was determined by DNA sequence analysis (Center for Gene Research and Biotechnology, Oregon State University, OR), and by restriction enzyme analysis (destroyed *Acc*I site). The 1.01-kb fragment containing the βY297C mutation was re-inserted into pRA100 to form pRA130. The mutant βY297C:E381C/εS108C was constructed from a combination of the βY297C mutation with mutations in the DELSEED region of β (Glu-381 → Cys) and in ε (Ser-108 → Cys) to form the plasmid pRA144. Subcloning was carried out by ligation of the 6.74-kb *Eag*I/*Eag*I fragment of pRA130 (containing βY297C) to the 5.94-kb *Eag*I/*Eag*I fragment of pRA134 (containing βE381C/εS108C, [27]. The *unc*$^-$ *E. coli* strain AN888 was transformed with the plasmids pRA130 and pRA144 for expression of mutant ECF_1. The plasmid pAN45, and *E. coli* strains AN1460 (wild-type) and AN888, were the generous gift of Graeme B. Cox, The Australian National University, Canberra [16,29].

2.2. Growth of mutant strains and preparation of ECF_1

Analysis of growth yields was determined in minimal liquid media (0.1 M KPi 93 mM NH_4Cl, 0.8 mM Na_2SO_4, pH 7.5, 1.6 mM $MgCl_2$, 3.6 μM $FeSO_4$, 14.8 μM thiamine) supplemented with 100 mM succinate. ECF_1 was isolated and stored as previously described [9,30]. Immediately prior to use, enzyme was precipitated with 70% ammonium sulfate and transferred to an EDTA-containing buffer, either 50 mM Mops, pH 7.0, 0.5 mM EDTA, 10% glycerol (buffer A), 50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 10% glycerol (buffer B), or 50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 10% glycerol (buffer C), and passed through two consecutive centrifuge columns in order to remove loosely bound nucleotides, as previously described [16].

2.3. Equilibrium binding experiments with lin-benzo-ADP and calculation of apparent binding constants

Fluorescence measurements were performed using a SLM 8000 photon counting spectrofluorometer (SLM Inc., IL), at an excitation wavelength of 331 nm and an emission wavelength of 390 nm. Wild-type or mutant (βY297C) ECF_1 in buffer B was supplemented with $MgCl_2$ (2.5 mM) and diluted into buffer D (50 mM Tris-HCl, pH 8.0, 2.0 mM $MgCl_2$) prior to addition of *lin*-benzo-ADP (the generous gift of Alan E. Senior, University of Rochester Medical Center, Rochester, NY). Concentrations of *lin*-benzo-ADP were determined spectrophotometrically at 331 nm using an extinction coefficient of 9750 M^{-1} cm^{-1} [31]. ECF_1 (50 nM to 3.5 μM) was added to a given concentration of *lin*-benzo-ADP in separate experiments over a concentration range spanning three orders of magnitude (150 nM to 20 μM) and incubated at room temperature until binding equilibrium was reached (approx. 1 h). The concentrations of bound and free *lin*-benzo-ADP were then determined for each concentration of enzyme-bound nucleotide and the intrinsic binding constants calculated from a plot of [free *lin*-benzo-ADP] vs. [bound *lin*-benzo-ADP]/[F_1] as previously described in Weber et al. [32]. A curve fit which assumed three independent binding sites was applied using the KaleidaGraph™ data analysis and graphics program for personal computer, based on Eq. 1: number of ligands bound/mol $F_1 = C/(K_{d1} + C) + C/(K_{d2} + C) + C/(K_{d3} + C)$, where, K_{d1}, K_{d2}, and K_{d3} = intrinsic binding constants for nucleotide binding sites 1, 2 and 3, respectively, and C = [free *lin*-benzo-ADP] (see also Refs. [32,33]).

2.4. Chemical modification reactions and determination of ATPase activity

Reactions of mutant (β297C) and wild-type ECF1 (2 μM) with DCCD (500 μM), NEM (200 μM) and CM

(200 μM) were performed in buffer A supplemented with 5 mM ATP (DCCD) or 5 mM ATP + 5.5 mM $MgCl_2$ (NEM and CM), for 2–3 h at 23°C. Reaction with NbfCl was performed at either pH 7.5 (buffer C, wild-type) [23] or pH 7.0 (buffer A, mutant) [34] for 1 h at 30°C, then excess reagent was removed by column centrifugation. Aliquots of NbfCl-modified enzyme were removed for treatment with DTT (20 mM, 2 h), activity measurements and analysis by SDS-PAGE. Untreated aliquots of mutant and wild-type ECF_1 were subjected to the same buffer and incubation conditions to control for any loss of activity throughout this period of time. Aliquots of each reaction mixture (5 μg/ml) were withdrawn at the conclusion of incubation periods and the ATPase activity assayed immediately using an ATP regenerating system at pH 8.0, 2 mM ATP and 2 mM $MgCl_2$ (modified from Ref. [35]). In a separate experiment, NaN_3 (final concentration 2 mM) was added directly to aliquots of unmodified F_1 in the assay cuvette in order to determine the inhibitory effect of NaN_3 on ATPase activity. Aliquots of both CM-modified and NbfCl-modified enzyme were quenched with 10 mM NEM then subjected to SDS-PAGE (in the absence of reducing agent) and UV illumination of separated β subunits in order to qualitatively confirm the covalent incorporation of reagent.

2.5. Incorporation of [^{14}C]NEM and calculation of kinetic constants

Mutant (β297C) ECF_1 (1 μM) was incubated with [^{14}C]NEM (DuPont NEN: spec. act. 40.0 mCi/mmol), at either 20 μM or 200 μM, in buffer A, in the presence or absence of various nucleotides, for varying periods of time up to 3 h at room temperature. Nucleotide was added to give aliquots containing 5 mM ATP + 5.5 mM $MgCl_2$ ($ATP + Mg^{2+}$), 5 mM ATP + 0.5 mM EDTA (ATP + EDTA), 5 mM ADP + 5.5 mM $MgCl_2$ + 5 mM NaH_2PO_4 ($ADP + Mg^{2+} + P_i$), and 5 mM AMP-PNP + 5.5 mM $MgCl_2$ (AMP-PNP + Mg^{2+}). For determinations performed in the absence of added nucleotide, the reaction was carried out in buffer A (EDTA). Following 15–30 min equilibration at room temperature, 200 μM [^{14}C]NEM (2.9 mM stock in Me_2SO, spec. act. 40.0 mCi/mmol) was added to aliquots of known protein concentration, both in the presence and absence of 2% SDS. Aliquots were withdrawn at specific time intervals (15 s through 30 min) and quenched at each time point by the addition of 10 mM NEM. These were then applied to a 10–18% linear gradient SDS-PAGE for determination of the molar incorporation of [^{14}C]NEM into β subunits [30,36]. Briefly, samples labeled in SDS were used to calculate how many c.p.m. would be expected in any Cys when one mol of [^{14}C]NEM is incorporated. This determination is based on there being 4 Cys in the α subunit and 2 in the γ subunit, all of which are available for reaction in the detergent-treated samples. The rate of incorporation of NEM into βCys-297 was described by kinetic rate constants which assume pseudo-first order reactions of one, two or three independently reacting Cys residues as previously described [33,37].

2.6. $CuCl_2$ induced cross-link formation in the mutant βY297C:E381C/εS108C

Prior to induction of disulfide bond cross-links, selective modification of Cys-297 in the mutant βY297C:E381C/εS108C, in only 1 mol of β subunits, was carried out by reaction of ECF_1 (10 μM) with [^{14}C]NEM (20 μM) in the presence of ATP + Mg^{2+}. After incubation for 1 min, further incorporation of NEM was stopped and excess reagent and nucleotides removed by passage through two consecutive centrifuge columns equilibrated in buffer A. The eluate was divided into three aliquots, one of which received no further treatment and was used to calculate molar incorporation of NEM; a second aliquot was treated with 100 μM $CuCl_2$ (in the presence of 2.5 mM $MgCl_2$) to induce disulfide bond cross-links, while a third aliquot was reacted with 200 μM [^{14}C]NEM, in the presence of 2% SDS, for calibration purposes. The incorporation of NEM into β subunits (mol/mol F_1), i.e., both free β (including a small incorporation into β-δ) and cross-linked β subunits (β-ε and β-γ), was determined from gel slices isolated by SDS-PAGE (in the absence of DTT) as previously described [37].

2.7. Other procedures and materials

The BCA protein assay was from Pierce. SDS-PAGE was performed with a 4% stacking gel and 10–18% linear gradient separating gel [38]. NEM, DCCD, and NbfCl were from Sigma, and CM from Molecular Probes.

3. Results

3.1. The βTyr297 → Cys mutation gives reduced growth and altered ATPase activity

The mutant βY297C proved to grow only poorly relative to the isogenic wild-type strain (pRA100/AN888) in minimal liquid media supplemented by succinate, indicating an impairment of oxidative phosphorylation. Thus at 18 h when the optical density of wild-type had reached 1.8 (the maximum), that of the mutant was 0.6. The ATPase activity of ECF_1 isolated from the mutant was also markedly reduced, i.e., 0.15–0.25 μmol ATP hydrolyzed $min^{-1} mg^{-1}$, compared with wild-type ATPase activity of 16–20 μmol ATP $min^{-1} mg^{-1}$, when assayed at pH 8.0 in the presence of 2 mM ATP and 2 mM $MgCl_2$. While this ATPase activity of ECF_1 from the mutant βY297C is only around 1% of wild-type, the rate is still approx. 10^4-fold higher than the reported rate of uni-site catalysis by ECF_1

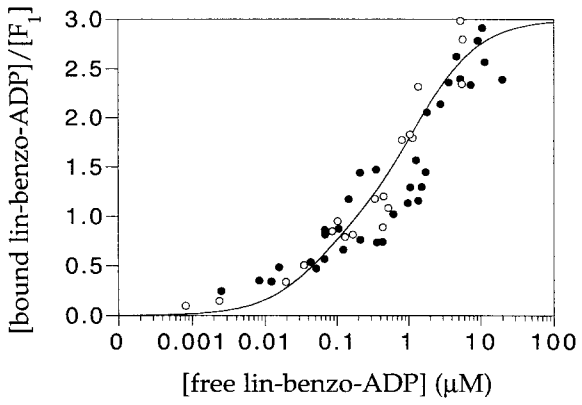

Fig. 1. Equilibrium binding of *lin*-benzo-ADP to wild-type and mutant (βY297C) ECF_1. The results of several equilibrium binding experiments are shown in which wild-type (open circles) or mutant (closed circles) ECF_1 (0.05–3.5 μM) were incubated with *lin*-benzo-ADP (0.15–20 μM) in buffer D until no further decrease in fluorescence intensity relative to free *lin*-benzo-ADP was observed, i.e., equilibrium conditions were reached. The relative emission intensity was recorded for each concentration of enzyme-bound *lin*-benzo-ADP and intrinsic binding constants calculated from computer-generated curve fits (solid line, wild-type only shown) as described in the text.

[39], implying that the mutant enzyme is competent in (slow) multi-site catalysis.

Higher concentrations of DCCD (500 μM) inhibited the ATPase activity of the mutant βY297C to less than 0.05 μmol ATP $min^{-1} mg^{-1}$, i.e., by approx. 75% while inhibition by NaN_3 (2 mM) was 45% cf. 90% for wild-type enzyme. The inhibitory effect of these two reagents, both of which affect multi-site but not uni-site catalysis, is further evidence that the low but significant ATPase activity of the mutant βY297C involves cooperative, multi-site enzyme turnover [40–42]. The low activity of the mutant is not a result of an altered subunit composition. SDS-PAGE of ECF_1 from the mutant βY297C showed the same subunit pattern as wild-type enzyme. There was no major loss of γ, δ, or ε subunits (see later). Moreover, the low activity does not appear to be due to an altered affinity for nucleotide, based on experiments with *lin*-benzo-ADP. This fluorescent analog has been shown to bind to wild-type ECF_1 with a stoichiometry of 3 mol/mol F_1 [32,43]. Fig. 1 compares data from equilibrium binding experiments using the mutant βY297C and wild-type ECF_1. No significant difference in nucleotide binding between the mutant and wild-type enzyme was detected. The binding constants obtained from computer curve fitting were $K_{d1} = < 0.15$ μM, K_{d2} and $K_{d3} = 1.4$ μM (wild-type), and $K_{d1} = <$ 0.15 μM, K_{d2} and $K_{d3} = 2.7$ μM (mutant), in both cases, values slightly higher than, but comparable to published values [32].

3.2. NbfCl reacts with and activates the mutant βY297C

To further explore the inhibitory effect of the Cys for Tyr replacement at position β297, the mutant βTyr-297 was reacted with different sulfydryl reagents to vary the side chain group with respect to size and hydrophobicity. The smallest reagent tested, NEM, increased ATPase activity approx. 5-fold. NbfCl also modified the mutant βY297C. This reagent reacts equally well with Cys residues as with Tyr [34]. While NbfCl modification of Tyr at β297 inhibited activity by 95%, reaction of the Cys at the same position with this reagent gave a six-fold increase of ATPase activity to 1.5 μmol ATP hydrolyzed $min^{-1} mg^{-1}$ protein, which is approx. 13% of the activity of wild-type enzyme. DTT treatment to release NbfCl from the Cys [44] caused loss of activity back to the rate obtained with unmodified mutant βY297C. In contrast to the activation obtained with NEM or NbfCl, the bulky maleimide CM reacted with βCys-297, (based on fluorescence labeling of the β subunit, result not shown), but the modification had no effect on activity.

3.3. Asymmetry of the β subunits based on reactivity of βCys-297 to NEM

Introduction of a Cys for a Tyr at position 297 produced a site for chemical modification in all three β subunits, the reaction of which is a measure of β subunit equivalency. To examine the environment of Cys-297 in the different β subunits, ECF_1 from the mutant enzyme was incubated with [^{14}C]NEM (20 or 200 μM) for different lengths of time, the reaction quenched with cold NEM (20 mM), and then the incorporation of radioactivity analyzed by SDS-PAGE. As described previously, [^{14}C]NEM labeled the α subunit to a small extent and the δ subunit of ECF_1 to 0.7–0.9 mol/mol F_1 when wild-type enzyme was examined [36]. Reaction of the mutant ECF_1 with low concentrations of [^{14}C]NEM labeled the α and δ subunits, as in wild-type enzyme. In addition, there was labeling of the β subunit by modification of the Cys introduced at residue 297.

One mol of NEM per mol F_1 was incorporated into the β subunits very rapidly in all experiments. The kinetic rate constant for reaction of this first site was 2200 $M^{-1}s^{-1}$ in the presence of ATP + Mg^{2+}, 1500 $M^{-1}s^{-1}$ in the presence of ADP + Mg^{+2} + P_i, and 1370 $M^{-1}s^{-1}$ in the presence of ATP + EDTA. The constants were calculated from computer-fitted curves such as shown in Fig. 2, where each point represents the mean of 3–4 different experiments. The coefficient of variation between experiments was calculated to be approx. 15%. These kinetic rate constants only approximate the second order rate constant, as they are determined at only one or two concentrations of NEM. They compare with a (real) second order rate constant of 1600 $M^{-1}s^{-1}$ for the reaction of NEM with free cysteine [45], which suggests that Cys-297 of one of the three β subunits is exposed to solvent and readily available for reaction under all nucleotide conditions. The reaction of Cys-297 of a second β subunit varied much more dramatically with nucleotide conditions as evidenced

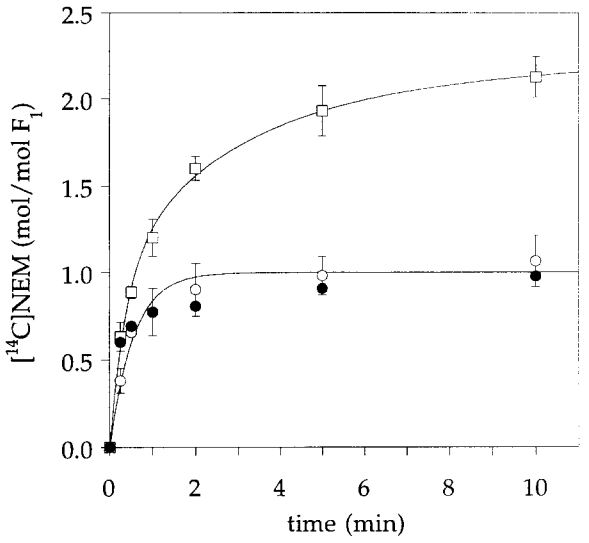

Fig. 2. Incorporation of [14C]NEM into β subunits of mutant ECF1 (βY297C) under different nucleotide conditions. The rates of incorporation of [^{14}C]NEM into βCys-297 were determined over a 60-min time course (10 min shown) by reaction of ECF_1 (1 μM) with 20 μM [^{14}C]NEM in buffer A supplemented with nucleotides, ATP + Mg^{2+} (squares), ADP + Mg^{2+} + P_i (open circles), or AMP-PNP + Mg^{2+} (closed circles), as described in the text. The mean of 3–4 independent experiments is plotted, with error bars representing 1 standard deviation. The solid lines are computer-generated curve fits from which kinetic rate constants were determined for each nucleotide condition assuming three independently reacting Cys residues (ATP + Mg^{2+}), or a single reactive site (ADP + Mg^{2+} + P_i).

in Fig. 2. Thus a second mol of NEM was incorporated into the mutant enzyme fairly rapidly in the presence of ATP + Mg^{2+}, ($k_2 = 315\ M^{-1}s^{-1}$). In ATP + EDTA this rate was 40 $M^{-1}s^{-1}$, while in the presence of ADP + Mg^{+2} + P_i or AMP-PNP + Mg^{+2} reaction of NEM into a second Cys was negligible. With a high concentration of [^{14}C]NEM (200 μM) for prolonged times (i.e., 2–3 h), the third copy of Cys-297 could be modified (rate constant around 15 $M^{-1}s^{-1}$) in ATP + Mg^{+2}. This site was unreactive under all other nucleotide conditions.

In a second set of experiments, NEM incorporation into different β subunits was examined using the mutant βY297C:E381C/εS108C. The distribution of [^{14}C]NEM between the different β subunits was obtained by reacting ECF_1 from this mutant with 20 μM reagent. An incubation time of 1 min was used, in which close to one mol of reagent was incorporated (see Fig. 2), in order to preferentially label only the fastest reacting copy of βCys-297. Under these same conditions, the labeling of β Cys 381 and ε Cys 108 in the double mutant βE381C/εS108C was less than 0.1 mol per Cys residue (result not shown). Samples were then treated with $CuCl_2$ to cross-link one β subunit to γ (β_γ) and a second to ε (β_ε) (Fig. 3A). As shown in Fig. 3B, most of the first mol of [^{14}C]NEM to be incorporated into the enzyme was in β_{free}, i.e., that β subunit not associated with the γ or ε subunits. As a portion of β_{free} becomes cross-linked to the δ subunit [27,37], the extent of incorporation into this β subunit was determined as the sum of the [^{14}C]NEM in both uncross-

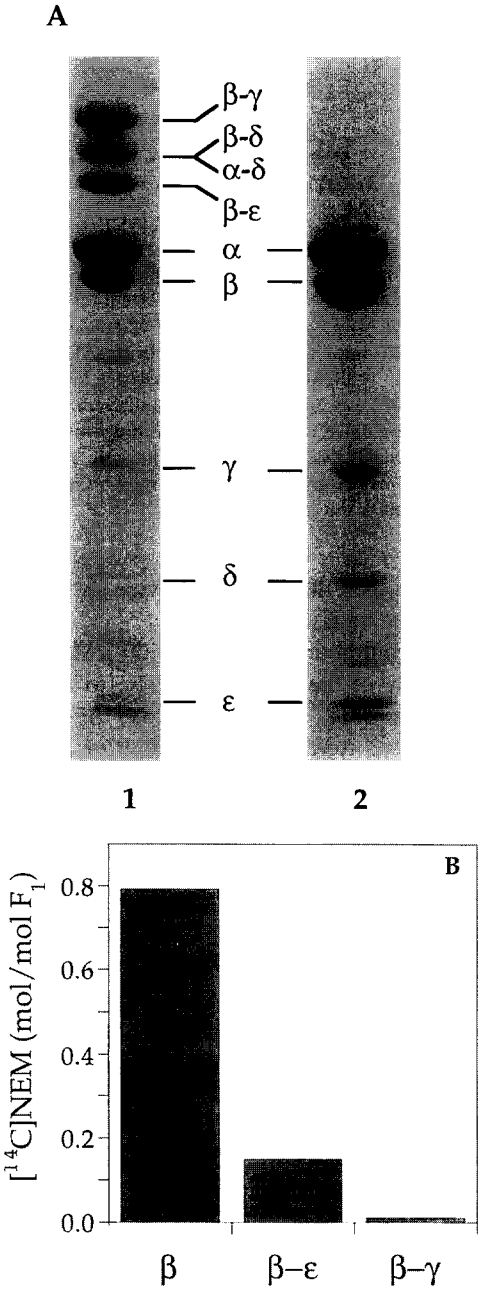

Fig. 3. Incorporation of 1 mol of [14C]NEM into βCys-297 of mutant ECF1 (βY297C:E381C/εS108C) followed by cross-linking. Mutant ECF_1 (10 μM) (βY297C:E381C/εS108C) was labeled with [^{14}C]NEM (20 μM) for 1 min in buffer A supplemented with ATP + Mg^{2+} and disulfide bond cross-links induced between β subunits and γ, δ, and ε subunits, as described in the text. Aliquots of cross-linked (lane 1) and non-cross-linked enzyme (lane 2) (50 μg per lane) were subjected to SDS-PAGE in the absence of DTT and stained with Coomassie brilliant blue R. The molar incorporation of [^{14}C]NEM (mol/mol F_1) into individual β subunits–β subunit (β_{free}), and cross-linked subunits, β-γ (β_γ), and β-ε (β_ε) was determined. A small amount of incorporation into the β-δ cross-link product was added to β_{free}, as described in the text.

linked β and in the β-δ product. There was a small amount of labeling of β_ϵ but no labeling of β_γ. A similar distribution of label was observed when the experiment was carried out with NbfCl (at pH 7.0), and the incorporation of label monitored by UV illumination of β subunits separated by SDS-PAGE (result not shown).

When the length of time of reaction of the mutant enzyme with [^{14}C]NEM prior to cross-linking was increased to 5 min or more to ensure incorporation of closer to 2 mol of reagent, the yield of cross-linked products dropped significantly. This is because of NEM modification of β Cys 381 and ϵ Cys 108, as indicated by experiments with the double mutant βE381C/ϵS108C (result not shown). However, there was still little or no incorporation of [^{14}C]NEM (less than 0.05 mol) in that β-γ cross-linked product formed (yield 50–60%), confirming that it is the reaction of Cys 297 of β_ϵ which is most influenced by nucleotide occupancy.

4. Discussion

Modification of βTyr-297 by NbfCl has been shown to inhibit the ATPase activity of F_1-ATPases [20], implying an important role of this residue. As Tyr-297 is the site of binding of 8-azido ATP [21], it was suggested that this residue is in the catalytic site and involved in substrate binding [46]. In contrast, Odaka et al. [26] reported that Tyr-307 (the equivalent residue to Tyr-297) can be changed to a Cys in TF_1 by site-directed mutagenesis without affecting activity.

We have now converted Tyr-297 to a Cys in ECF_1 and find that this mutation dramatically affects activity, giving an enzyme with only around 1% of the ATPase activity of wild-type. The Cys for Tyr change did not affect nucleotide binding based on equilibrium binding studies with *lin*-benzo-ADP. This result would be predicted by the recent X-ray structure determination of MF_1, which shows Tyr-311 (equivalent of Tyr-297) approx. 5 Å from the nucleotide binding site [10].

Our data indicate that the mutant βY297C retains cooperative or multi-site ATPase activity, albeit low, which is inhibited by DCCD and azide. The ATPase activity of the mutant enzyme could be increased to as high as 13% of that of wild-type by modification of the introduced Cys by NbfCl, which added to the bulk and increased the hydrophobicity of the side chain. The reaction of NbfCl with Cys-297 was reversed by reaction with DTT, confirming that the reagent had reacted with the Cys. CM also reacted with Cys-297, but failed to activate the enzyme, indicating that the size and/or shape of this side chain is important. Parsonage et al. [47] have shown that the mutation βY297F has only minimal effect on activity, supporting the idea that hydrophobicity rather than the side chain function is critical. Tyr-297 is at the carboxy terminus of β-sheet 7 of the catalytic site domain and leads into an extended loop (residues 298–305) that binds the γ subunit [10]. It may be that a relatively small hydrophobic side chain is required to optimize the interaction of this region with the γ subunit, and at the same time, allow the movement of the γ subunit that occurs during the catalytic cycle.

Modification of the Cys at 297 in the mutant ECF_1 by NEM was shown to occur differently with the three β subunits. Cys-297 of one β subunit reacted very rapidly, that of a second β subunit was very much slower, while the reaction in the third β subunit was negligible. This asymmetry of the β subunits was observed when the catalytic sites were empty of nucleotide and with ATP or ADP bound. Experiments with the mutant βY297C:E381C/ϵS108C, in which $CuCl_2$ induces disulfide bond formation between one β and γ and a second β and ϵ, allowed us to distinguish the different β subunits by their interaction with the single copy subunits. Labeling of this mutant, followed by cross-linking, located the Cys-297 that reacts most readily with NEM or NbfCl in β_{free}, i.e., that β subunit which does not become linked to either γ or ϵ subunit by the $CuCl_2$ treatment. The rate of NEM incorporation into this site was in the same range observed for free cysteine in solution, indicating that Cys-297 in β_{free} is exposed to solvent.

The second site to react with NEM is located on β_ϵ. Cys-297 in this β subunit was partly shielded and its reaction very much nucleotide-dependent. Its modification was quite fast in ATP + Mg^{2+}, but essentially non-existent in ADP + Mg^{2+} or AMP-PNP + Mg^{2+} under the experimental conditions employed. Given the significant, if low, ATPase activity of the mutant βY297C, and activation by incorporation of NEM, the enzyme will be turning over during the reaction time (30 min) of the reagent, when there is 5 mM ATP present. Only under these turnover conditions is there reaction of two and probably three β subunits at Cys-297. However, such continuous enzyme turnover did not equalize the sites; they retained different rates of NEM modification. This result is hard to explain by a binding change mechanism (e.g., Boyer [4]), in which the β subunits cycle through each of the different possible conformations with every two or three turnovers of the enzyme during bi-site or tri-site catalysis.

Recently Schmidt and Senior [48] have described experiments in which they modify a mutant of ECF_1 βS339C with [^{14}C]NEM. They find that one mol of reagent reacts with Cys-339 rapidly in an apparently nucleotide independent manner, while the second mol of reagent to bind is nucleotide-sensitive, being rapid if there is enzyme turnover during the labeling (ATP + Mg^{2+}), slower in ADP + Mg^{2+}, and extremely slow in AMP-PNP + Mg^{2+}. These authors do not distinguish the sites of reaction, but it is likely that the first Cys-339 to react is in β_{free} and the second in β_ϵ. The explanation of the nucleotide dependence observed here for Cys-297, and by Schmidt and Senior [48] for Cys-339, is not obvious at present, but may involve shielding of these residues in β_ϵ by interactions of

the γ subunit, which are altered by turnover and movements of the γ subunit.

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