Protein Synthesis

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Reconstitution of the Catalytic Core of F-ATPase $(\alpha\beta)_3\gamma$ from *Escherichia coli* Using Chemically Synthesized Subunit γ**

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ATP synthesis from ADP and P_i accounts for the vast majority of ATP consumed by higher organisms. The endergonic reaction requires both, catalysis and the input of free energy, which is provided by the ion motive force across the ATP synthase (F_oF_1) containing coupling membrane. Ion flux through the intramembrane portion F_o results in a unidirectional mechanical rotation of the rotor portion of the enzyme. By means of elastic coupling this liberates spontaneously formed, but tightly bound ATP from the reactive sites. In this way the electrochemical potential of the "coupling ion" is mechanically coupled to the chemical reaction. For recent reviews see references [1–4]. ATP synthase works fully reversibly: Upon the expenditure of ion motive force it synthesizes ATP, upon hydrolysis of ATP it generates an ion motive force. These two modes are strictly coupled to clockwise or counterclockwise rotation. It is conceivable that the direction of rotation is linked to the stereochemical configuration of the enzyme.

A coupling of configuration with function has been demonstrated in the past for the HIV protease,[5] whereas a mere physicochemical interaction, for example, between an antifreeze protein and water, was shown to be independent of the configuration.[6] ATP synthase is an interesting case because of its mechanical intermediate in catalysis. F_oF_1 consisting entirely of D-amino acids would be expected to have its direction of rotation/function relationship inverted. Since the central rotor portion of the enzyme, subunit γ, can rotate in both directions, the mirror-image version of γ might be compatible with the enzyme's function, since the molecular surfaces of L-γ and D-γ are highly similar. Whether the F-ATPase catalytic core complex consisting of L-type subunits α and β plus synthetic D-γ retains activity and, if so, also what direction of the rotation/function relationship is in effect, can be decided only by experiment.

Such a venture would require the chemical synthesis of the 286 residue D-enantiomer of γ, followed by reconstitution of the core enzyme. The synthetic tools for this goal were developed and refined during the last decades.[7,8] As a first step in this direction we have synthesized the F-ATPase subunit L-γ from *Escherichia coli* (herein called "γ_{synth}"; for deviations from the native sequence see below) and successfully reconstituted it along with native α and β into the core ATPase complex, $(\alpha\beta)_3\gamma_{synth}$.

In view of its size, we divided subunit γ into six peptides of roughly equal length (38–58 residues); these were expected to be smoothly synthesizable and compatible with the requirements of the native chemical ligation but at the same time remaining as isosteric as possible (Scheme 1).

Cost, yield, and purity considerations pointed to Boc rather than Fmoc chemistry for solid-phase peptide synthesis. To construct the entire sequence, in view of the documented examples we chose consecutive chemical ligation[7] rather than the convergent approach[10–12] (Scheme 2). The one-pot approach[9] was attempted with the first three ligations, but in our hands gave unsatisfactory results.

Native chemical ligation (NCL) yields native peptide bonds but calls for a thioester at the C terminus of peptide *i* and a cysteine residue at the N terminus of peptide $i+1$.[7] The residue carrying the thioester instead of the standard carboxy group must neither be branched at Cβ nor be an aspartate or glutamate.[13] Thus five sequence alterations were unavoidable (N59C, K108C, S146C, S193C, and S237C). Substitution of prexisting cysteine residues (C87A and C112A) and the substitution K108C had been carried out earlier in order to tailor EF_1 for microvideography of rotation experiments.[14] Since C108 ultimately would continue to be required for microvideography and in view of the potential side reactions of yet another chemical treatment, desulfurization of the introduced cysteine residues was not considered. Rather γ with these seven point mutations was generated by polymerase chain reaction and checked in a control experiment. It was shown to behave just as the wild-type subunit in terms of biosynthesis, cell growth, purification, ATPase activity, and rotation of immobilized core F-ATPase[14,15] (data not shown).

Apart from the above sequence alterations, four more, which had not been foreseen, turned up during synthesis.

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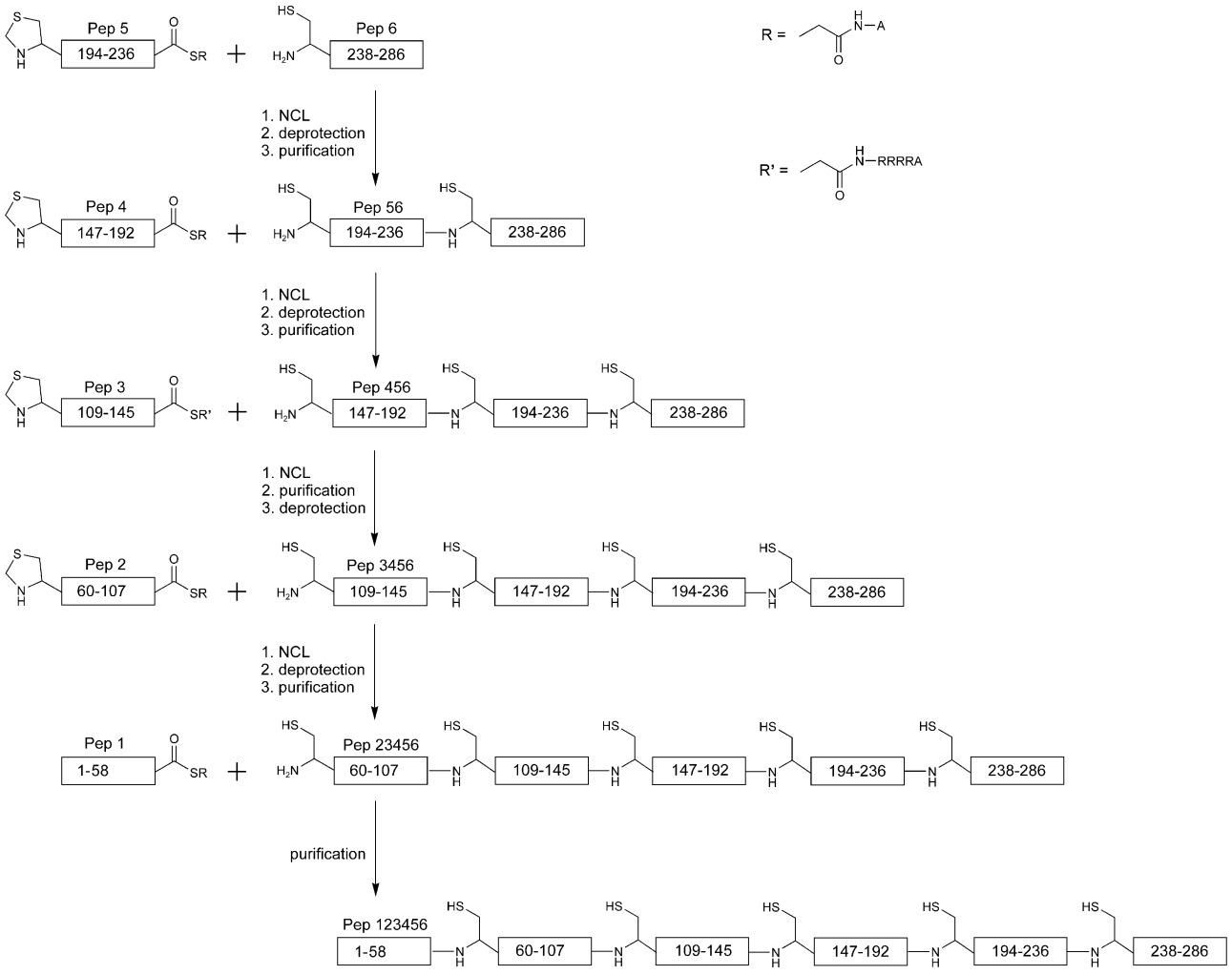
Peptide	Number of residues	Sequence	Mass [Da]
1 (1-58)	58	AGAK**D**IRSKIASVQNTQKITKAMEMVAASKMRKSQDRMAASRPYAETMRKVIGHLAHG**XA**	6516.6
2 (59-107)	49	**Z**LEYKHPYLEDRDVKRVGYLVVSTDRGL**A**GGLNINLFKKLLAEMKTWT**A**X**A**	5825.8
3 (108-145)	38	**Z**GVQ**A**DLAMIGSKGVSFFNSVGGNVVAQVTGMGDNPSL**XRRRRA**	4524.2
4 (146-192)	47	**Z**ELIGPVKVMLQAYDEGRLDKLYIVSNKFINTMSQVPTISQLLPLPA**XA**	5419.5
5 (193-236)	44	**Z**DDDDLKHKSWDYLYEPDPKALLDTLLRRYVESQVYQGVVENLA**XA**	5386.0
6 (237-286)	50	**C**EQAARMVAMKAATDNGGSLIKELQLVYNKARQASITQELTEIVSGAAA**A**	5222.9

Scheme 1. Sequences of synthetic peptides of the F-ATPase subunit γ from *E. coli*. The N-terminal Met residue as given, for example, in the UniProt database (http://www.uniprot.org, entry P0ABA6) is not present in the mature chain and hence not included here. The sequence of γ was renumbered accordingly. Deviations in amino acid sequence from the wild-type protein are highlighted in boldface. Upon cleavage of the resin, residue X yields the thioester (*S*-trityl-β-mercaptopropionic acid) derivative of the preceding residue (peptides 1–5). Z indicates protected cysteine, introduced via 1,3-thiazolidine,[9] which after ligation of two peptides and deprotection is converted into cysteine (peptides 2–5). Peptide 6 therefore contains an N-terminal-unprotected cysteine residue right off. The C-terminal alanine residues (peptides 1, 2, 4, 5) and the RRRRA sequence at the C terminus of peptide 3, required for purification, are removed during native chemical ligation.

These comprised: 1) E5D in peptide 1, which was caused by an error in our database and noticed only shortly before completion of the ligations. It was ignored in view of the very considerable efforts necessary to correct this error at that point. 2) W203 in peptide 5 originated from a different kind of protected amino acid (*tert*-butyloxycarbonyltryptophane, Boc-Trp-CHO) rather than the reagent used for W105 in peptide 2 (Boc-Trp-OH). The former was not deprotected by the applied procedure but retained its formaldehyde group (see the Supporting Information) and thus represented the most significant deviation from the ribosomally synthesized protein. Fortunately, W203 is located at the very periphery of subunit γ, beyond the contact surfaces with the $(\alpha\beta)_3$ hexagon (pdb: 3oaa).[16] There it cannot interfere with rotation and hence ATPase activity. 3) It also turned out that D107 had to be exchanged for an alanine residue. Owing to the charged side chain of aspartate, a side



Scheme 2. The total chemical synthesis of the F-ATPase subunit γ from *E. coli* .

product arose during chemical ligation which decreased the total yield significantly.[13] 4) The substitution V286A was necessary because the valine–PAM linker did not react quantitatively with the resin. This resulted in portions of the growing chain to bind directly and irreversibly to the resin. As up to twelve C-terminal amino acid residues of *E. coli* F_1-γ can be deleted without completely destroying activity,[17] this latter change was expected to have a negligible effect.

The relative robustness of γ against sequence alterations[2] is underlined by its poor sequence conservation, which in view of its predominantly mechanical role is not that surprising. Here, this proved to be quite favorable with respect to the total of eleven sequence alterations outlined above.

After separation from the resin and deprotection with HF, the resulting crude peptides were purified by high-pressure liquid chromatography at C4-reversed-phase matrices with a trifluoroacetic acid (TFA)/acetonitrile/water buffer system. The elution protocol was optimized for each peptide by analytical runs with 1 mg samples, followed by preparative runs.[18] The identity of the eluted peptides was confirmed by electrospray-ionization mass spectrometry (for details see the Supporting Information).

The five ligations required for the construction of the full-length chain were performed according to the standard protocol; at set intervals we characterized the ligation products by mass spectrometry, checked the time course of the ligation, and then purified the products by liquid chromatography. The products of the first two ligations (peptides 5 + 6 => peptide 56 and peptides 4 + 56 => 456) were deprotected directly in the ligation mixture after a total ligation time of 23 h. In the following three ligations the crude ligation mixture was first purified by chromatography and then deprotected by methoxyamine hydrochloride (MAH) without subsequent removal of MAH. Peptides 3 and 3456 otherwise were lost in chromatography. This procedure, however, could not be applied to the last ligation (peptides 1 + 23456). Peptide 1 unexpectedly reacted quantitatively with MAH (signal at *m/z* [(expected peptide mass)] + 29 Da, data not shown) and thereafter did not ligate. Therefore the complete ligation mixture had to be rechromatographed in this case, unfortunately resulting in further loss of ligation product.

The side reaction must have affected the thioester, likely caused by the specific sequence of peptide 1 (..AHGXA, cf. Scheme 1). If so, this would compromise the general applicability of the 1,3-thiazolidine (Thz) protection group especially in one-pot ligations[9] and call for alternative approaches. In future preparative work two unwanted side effects therefore should be kept in mind: loss of deprotected peptide residue(s) in chromatography and the observed side reaction with MAH.

Table 1 summarizes the yields and Figure 1 summarizes the chromatographic analyses of the purified ligation products, respectively. For reconstitution, isolated subunits α, β, and γ were mixed in the presence of ATP, kept at room temperature for 60 min, and then gelfiltrated.[19] The peak containing the assembled enzyme was clearly discernible from the single subunits. The material was further concentrated by direct application to Ni-NTA chromatography and

Table 1: Yields of the peptide ligations. The total synthesis started with 115 mg of peptide 6 and 142 mg of peptide 5.

Starting peptide	Starting peptide	Ligation product	Yield mg | %
6	5	56	69 | 30
56	4	456	24.3 | 23.5
456	3	3456	2.6 | 8.5
3456	2	23456	0.33 | 10
23456	1	γ_{synth}	0.03 | 8

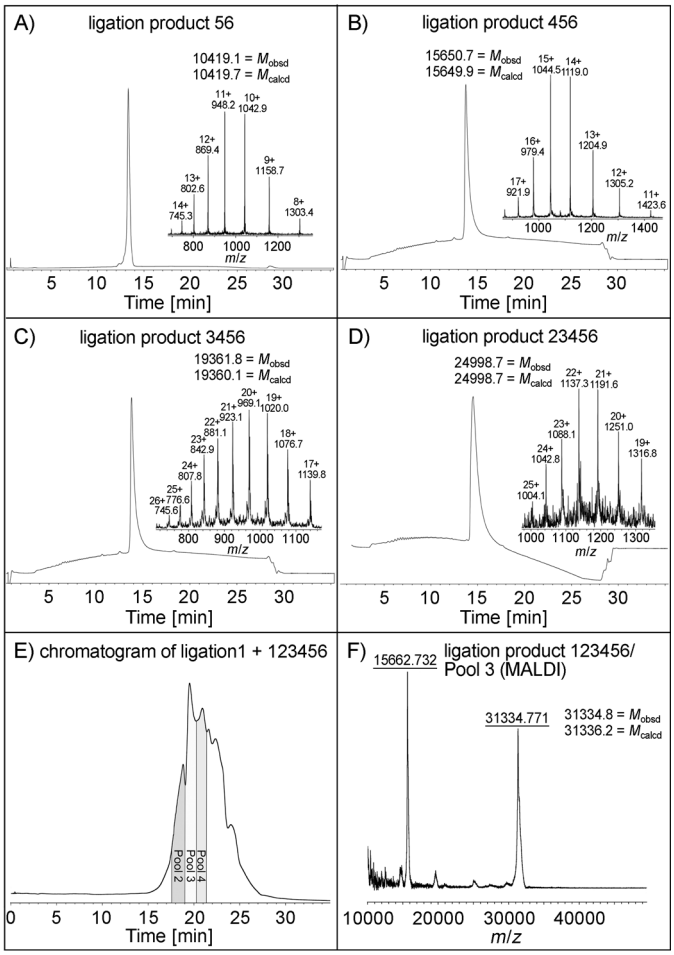


Figure 1. Chromatographic and mass spectrometric analyses of ligation products. In contrast to the other chromatographic separations, the chromatographic analysis of ligation product 1 + 23456 used a linear gradient of 30–70% buffer B in 80 min (40°C). Buffer A: 0.1% TFA in 10% acetonitrile/H_2O; buffer B: 0.08% TFA in 100% acetonitrile (part E). The complete peptide "123456" (Pool 3 = γ_{synth}) was lost in LC-ESI mass spectrometry and hence analyzed by MALDI mass spectrometry instead (F).

checked by SDS polyacrylamide gel electrophoresis and an ATPase assay. Figure 2 shows the chromatograms of core enzyme complexes assembled with either ribosomally or chemically synthesized γ.

The ATPase activities of the various reconstituted core enzymes are summarized in Table 2 (γ_{synth} originated from pool 3 of the chromatographic separation (cf. Figure 1 E); pools 2 and 4, which preceded or followed pool 3 in the same

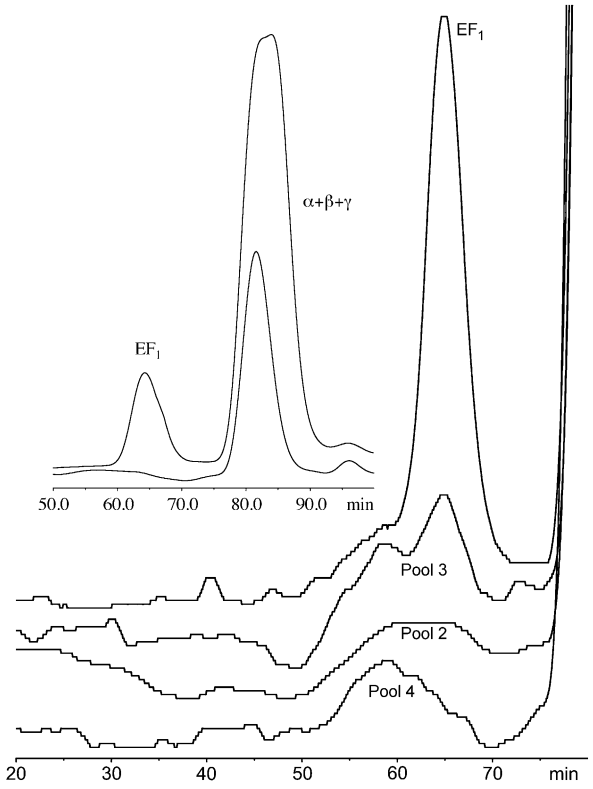


Figure 2. Gel-filtration of reconstituted core ATPase $(\alpha\beta)_3\gamma$. UV traces (280 nm) of small-scale preparative runs. GE Superdex 200, 16×600 mm, 0.5 mL min^{-1}. Main portion of the figure, upper trace: reconstitution with biosynthetically synthesized subunit γ_{nat}; next three traces: reconstitution with chemically synthesized γ_{synth}. Pools 2, 3, and 4 from the chromatographic separation of the final ligation product (cf. Figure 1 E) as indicated. The peak labeled "EF_1" contained about 6 μg of protein from a reconstitution with 90 μg each of subunits α and β plus 20 μg γ_{nat}; the equivalent peak from Pool 3 contained about 1.5 μg of protein from $\alpha+\beta+\gamma_{synth} = 23+23+5$ μg. The insert shows gel-filtrations of reconstituted F-ATPase core complex with and without subunit γ_{nat}. The peak labeled "EF_1" here contained 68 μg protein (reconstitution of 1 mg each of subunits α and β and 222 μg of γ).

Table 2: ATPase activities of EF_1 (freshly purified from EDTA extract) and reconstituted, purified EF_1 core complex $(\alpha\beta)_3\gamma$). Shown are the averages of the measurements. ATPase activity is expressed as μmol of phosphate generated by ATP hydrolysis per minute and mg enzyme.

Sample	ATPase activity (number of measurements)
EF_1	100±10 ($n=8$)
$(\alpha\beta)_3\gamma_{nat}$	98±6 ($n=3$)
$(\alpha\beta)_3\gamma_{synth}$	98±6 ($n=3$)

separation, did not yield active enzyme). Within their limits they were indistinguishable. Further details of the reconstitution procedure and additional data with respect to the yield of reconstituted enzyme are provided in the Supporting Information. The purity of the isolated subunits and (reconstituted) enzyme was confirmed by SDS electrophoresis (Figure 3).

The biologically active protein with 286 residues described herein currently represents the longest nonredundant polypeptide chain synthesized by chemical means. The major

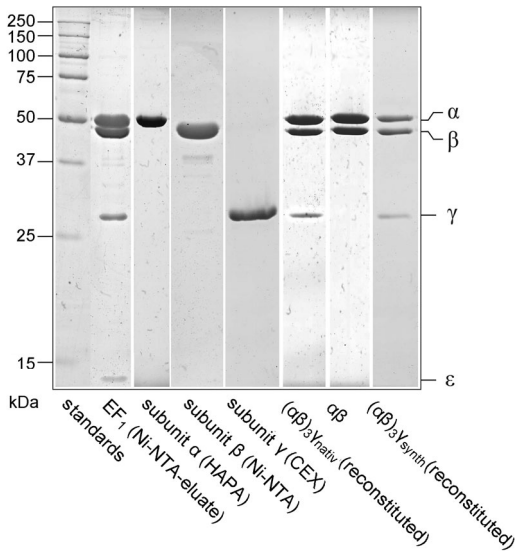


Figure 3. SDS polyacrylamide gel electrophoresis (12.5% Laemmli gel) of EF_1, subunits α, β, and γ, and reconstituted enzyme core complex with biosynthetically ($(\alpha\beta)_3\gamma_{native}$) or chemically ($(\alpha\beta)_3\gamma_{synth}$ with γ from pool 3) synthesized subunit γ. Sample αβ was a reconstitution setup without γ. 10 μg of protein was applied per lane except for $(\alpha\beta)_3\gamma_{synth}$ (reconstituted), where 3 μg was applied. For details, see the Experimental Section.

drawback of our synthesis is its poor yield. This was not caused by limitations in peptide availability. Rather the yields of the five consecutive ligations, especially the last three, dropped the total yield to only 0.005%. Owing to these circumstances, we responded to the low ligation yields simply by increasing the amounts of peptides to be ligated. In contrast to the recently described total chemical synthesis of tetraubiquitin,[20] in our case the drop in ligation yields may point to a dependence upon peptide length and possibly the number of cysteine residues. An optimization of the ratio of the number of ligations with respect to peptide length might be worth trying, since fewer ligations might outweigh lower yields with longer peptides. Also kinetically controlled ligation remains an experimental alternative to be tested. Despite the aforementioned problems and extremely low yield, the enormous preparative and analytical efforts inherent in the presented results have paid off in terms of a biologically active protein. We feel this to be quite encouraging in view of future efforts to synthesize proteins.

Experimental Section

EF_1 was extracted from membranes with EDTA and purified by anion-exchange and subsequent Ni-NTA chromatography. Subunit α was purified from dissociated EF_1 by Ni-NTA chromatography and subsequent hydroxy apatite chromatography. Subunit β was purified from dissociated EF_1 by Ni-NTA chromatography. Subunit γ was overexpressed in *E. coli* and purified by cation-exchange chromatography in the presence of 8 M urea. For reconstitution subunits were mixed in an approximate stoichiometric ratio of 3:3:1 and further processed as outlined in the Supporting Information.

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