One-step purification of the NADH dehydrogenase fragment of the *Escherichia coli* complex I by means of *Strep*-tag affinity chromatography

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Received 10 August 1999; received in revised form 30 September 1999

Abstract The proton-pumping NADH:ubiquinone oxidoreductase, also called complex I, is the first energy-transducing complex of many respiratory chains. Complex I of *Escherichia coli* can be split into three fragments. One of these fragments, the soluble NADH dehydrogenase fragment, represents the electron input part of complex I. It comprises the subunits NuoE, F and G and harbors one flavin mononucleotide and up to six iron-sulfur clusters. Here, we report the one-step purification of this fragment by means of affinity chromatography on StrepTactin. This was achieved by fusing the *Strep*-tag II peptide to the C-terminus of NuoF or NuoG. Fusion of this peptide to the N-terminus of either NuoE or NuoF disturbed the assembly of the NADH dehydrogenase fragment.

Key words: Complex I; NADH:ubiquinone oxidoreductase; NADH dehydrogenase fragment; *Strep*-tag II; Overexpression; *Escherichia coli*

1. Introduction

The proton pumping NADH:ubiquinone oxidoreductase, also called complex I, is the first of the respiratory complexes providing the proton motive force which is essential for the synthesis of ATP. Complex I couples the transfer of two electrons from NADH to ubiquinone with the translocation of four protons across the membrane [1–3]. Closely related forms of complex I are found in the respiratory chains of many bacteria and most mitochondria. In bacteria, the complex has a molecular mass of approximately 530 kDa and consists in general of 14 different subunits. Seven subunits are peripheral proteins including all subunits that bear the known redox groups of complex I, namely one flavin mononucleotide (FMN) and up to nine iron-sulfur (FeS) clusters [2,4]. The remaining seven subunits are very hydrophobic proteins and are predicted to fold into 54 α-helices across the membrane. Nothing is known about their function, but they are most likely to be involved in ubiquinone reduction and proton translocation [2–4]. The homologues of the 14 subunits together with some 27 additional subunits make up the mitochondrial complex, adding up to a molecular mass of approximately 1 MDa [1,2].

The genes of the *Escherichia coli* complex I are organized in the *nuo* operon (from NADH:ubiquinone oxidoreductase) [5]. The genes *nuoC* and *D* are fused to form one gene *nuoCD* leading to a complex consisting of 13 subunits [6,7]. By means of salt treatment and raising the pH, the isolated complex was split into a NADH dehydrogenase fragment, a connecting fragment and a membrane fragment [8]. The soluble NADH dehydrogenase fragment consists of the subunits NuoE, F and G, and harbors the binding sites for NADH and FMN and probably six FeS clusters, four of them being observable by EPR spectroscopy. This fragment catalyzes the oxidation of NADH in the presence of artificial electron acceptors like ferricyanide or hexammineruthenium and represents the electron input part of complex I [6]. Overexpression of *nuoB–G* in *E. coli* led to the overproduction of the fully assembled NADH dehydrogenase fragment. Expression of *nuoB* and *CD* is required for a proper assembly of the fragment, although the corresponding proteins are not present in the preparation [6].

In order to circumvent the time consuming preparation of this fragment using standard chromatographic methods we established a direct affinity chromatography based on engineered Nuo subunits. The *Strep*-tag II affinity peptide [9] was fused individually to the C- or N-terminus of the subunits of the NADH dehydrogenase fragment. Engineering the C-terminus of either NuoF or NuoG with the affinity peptide enabled us to purify the NADH dehydrogenase fragment in one step by means of chromatography on StrepTactin. Routinely 15–20 mg of a more than 95% pure protein complex was obtained within 3 h.

2. Materials and methods

2.1. Materials and strains

E. coli strains DH5α [10] and BL21 (DE3) [11], cloning vectors pUC 18, pACY184 (both Biolab, Schwalbach), pCRScript (Stratagene, Amsterdam) and the expression vector pET-11a (AGS, Heidelberg) were used. When required for maintenance of plasmids, ampicillin (100 μg/ml) and kanamycin (50 μg/ml) were added. All enzymes used for recombinant DNA techniques were from Pharmacia, Freiburg or Biolabs, Schwalbach.

2.2. Construction of expression vectors

Standard methods for molecular biology were used [12]. DNA sequencing was performed using the T7 sequencing kit (Pharmacia, Freiburg) [13]. The expression plasmid pET-11a/*nuoB–G* was constructed as described [6]. The *Strep*-tag II coding sequence 5′-GCTAGCTGGAGCCACCCGCAGTTCGAAAAA-3′ (IBA, Göttingen) [9] was introduced downstream of positions 2566 and 3064 of the *nuo* operon for N-terminal fusion with NuoE and NuoF and upstream of positions 3060, 4000 and 7179 of the *nuo* operon for C-terminal fusion with NuoE, NuoF, and NuoG, respectively, using standard two-step PCR methods (numbering according to [5]). First, inner and outer primers (Table 1) were used to amplify fragments upstream and downstream of the indicated positions including overlapping parts of the *Strep*-tag II coding sequence. Second, these fragments were combined and the outer primers were used to amplify a joined fragment including the entire *Strep*-tag II coding sequence. The resulting fragments were cloned in pET-11a and the constructs were verified by sequencing.

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0014-5793/99/$20.00
PII: S0014-5793(99)01341-1

2.3. Expression of the modified NuoB–G subunits

Competent *E. coli* cells BL21 (DE3) were transformed with pET-11a/*nuoB–G*/NuoE$_N$, pET-11a/*nuoB–G*/NuoE$_C$, pET-11a/*nuoB–G*/NuoF$_N$, pET-11a/*nuoB–G*/NuoF$_C$ or pET-11a/*nuoB–G*/NuoG$_C$. The indices N and C refer to the N- or C-terminal position of the *Strep*-tag II at the corresponding protein. The resulting transformants were grown, harvested and stored as described [6].

2.4. Purification of the NADH dehydrogenase fragment

All steps were carried out at 4°C. Cells (30 g) were resuspended in 55 ml 50 mM MES-NaOH, 50 mM NaCl, pH 6.6, 10 μg/ml DNase (Boehringer, Mannheim), protease inhibitor (Complete, Boehringer, Mannheim), and disrupted by a single passage through a French pressure cell (SLM Amicon) at 110 MPa. Cell debris and the cytoplasmic membranes were removed by centrifugation for 1 h at 250 000 × *g*. The supernatant was mixed with 0.6 mg avidin (Sigma, Munich) and applied to a 7 ml StrepTactin-Sepharose column (IBA, Göttingen) equilibrated in 50 mM MES-NaOH, 50 mM NaCl, pH 6.6. The column was washed with 20 ml buffer until no protein eluted from the column as judged by the absorbance at 280 nm. The NADH dehydrogenase fragment was eluted with 2.5 mM D-desthiobiotin (Sigma, Munich) in the same buffer as above at a flow rate of 0.6 ml/min. All other biochemical procedures and EPR spectroscopy were performed as previously published [6].

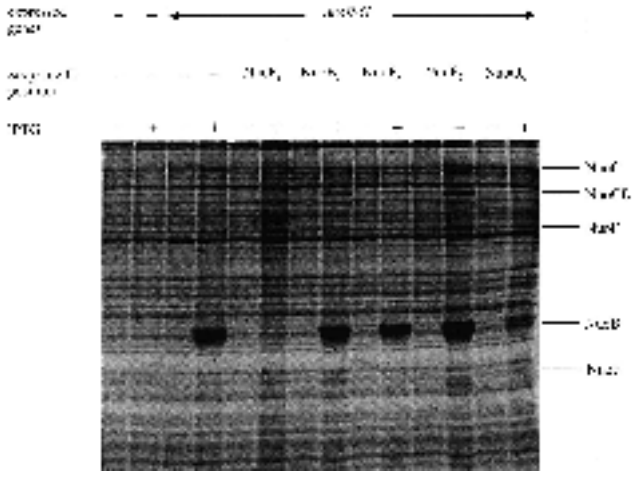

Fig. 1. Coomassie-stained SDS-PAGE of cell lysate of *E. coli* strain BL21(DE3) harboring the expression vector pET-11a with the cloned genes *nuoB–G*. The indices N and C denote the position of the *Strep*-tag II sequence on the corresponding subunit. Total cell protein with (+) or without (−) addition of IPTG to the growth medium is shown. The overproduced Nuo proteins are assigned.

3. Results

3.1. Overproduction of the tagged NADH dehydrogenase fragment

We constructed five different expression plasmids to enable the purification of the NADH dehydrogenase fragment of complex I by affinity chromatography. The *Strep*-tag II peptide binds with high affinity to the commercially available StrepTactin affinity material. However, it was not known whether the insertion of the additional 30 bp encoding the *Strep*-tag II peptide would influence the expression of the *nuo* genes, whether the fused peptide would disturb the assembly of the three-subunit complex or whether the tag would be accessible for affinity chromatography. Starting with the expression plasmid pET-11a/*nuoB–G* that harbors the genes *nuoB*, *CD*, *E*, *F* and *G* [6] the *Strep*-tag II coding sequence was introduced at the 3′ or 5′ ends of *nuoE*, *nuoF* and at the 3′ end of *nuoG*.

Overproduction of the *nuo* genes strongly depended on the position of the *Strep*-tag II sequence (Fig. 1 and Table 2). SDS-PAGE of total cell lysates from isopropyl β-D-thiogalactopyranoside (IPTG)-treated *E. coli* strain hosting plasmid pET-11a/*nuoB–G* showed the overproduced subunits NuoB, CD, E, F and G (Fig. 1). Cell lysates from IPTG-treated *E. coli* strain hosting plasmid pET-11a/*nuoB–G*/NuoE$_N$, in contrast, yielded no overproduced Nuo proteins. Most likely, the expression of the *nuo* genes was disturbed by the insertion of the *Strep*-tag II coding sequence. The *E. coli* strain hosting

Table 1
Oligonucleotides used in this study and length of the obtained PCR fragments

	Primer	Sequence (5′ → 3′)	Size of the PCR fragment (bp)
NuoE$_N$	outer primer 1	GCTTAAAGTGGAAGAGCTGC	
	outer primer 2	TTTTCTCGAGCGCCGCCTG	
	inner primer 1	GAACTGCGGGTGGCTCCAGCTAGCCATAATTAGCGGTCCACATCTG	
	inner primer 2	CTGGAGCCACCCGCAGTTCGAAAAACACGAGAATCAACAACCACAAAC	
			766
NuoE$_C$	outer primer 1	GTATTCAGGCGGCGCTCG	
	outer primer 2	CGATTTCGTCCGGAGACAG	
	inner primer 1	CTGGAGCCACCCGCAGTTCGAGAAATGAAAAACATTATCCGTACTCC	
	inner primer 2	GAACTGCGGGTGGCTCCAGCTAGCTTTATACCGCTCCAGCAGTTC	
			367
NuoF$_N$	outer primer 1	GTATTCAGGCGGCGCTCG	
	outer primer 2	CGATTTCGTCCGGAGACAG	
	inner primer 1	CTGGAGCCACCCGCAGTTCGAGAAGAAAAACATTATCCGTACTCCCG	
	inner primer 2	GAACTGCGGGTGGCTCCAGCTACTCATTTATACCGCTCCAGCAG	
			343
NuoF$_C$	outer primer 1	CTGTCTCCGGACGAAATCG	
	outer primer 2	CGGGATCCGTCCGCTCCGTTGACCTCG	
	inner primer 1	GAACTGCGGGTGGCTCCAGCTAGCCCAGCGCTCTTTCAGCAGG	
	inner primer 2	CTGGAGCCACCCGCAGTTCGAAAAATAACCGAATTTCGATTAACGCTC	
			1338
NuoG$_C$	outer primer 1	GCGATGAATTGTCACAGCGTG	
	outer primer 2	CGGGATCCAGTCTTCTTTAAAGAAC	
	inner primer 1	GAACTGCGGGTGGCTCCAGCTAGCTTGTTGTGCCTCCTTGAGATC	
	inner primer 2	CTGGAGCCACCCGCAGTTCGAAAAATGAGTTGGATATCACCGGAAC	
			514

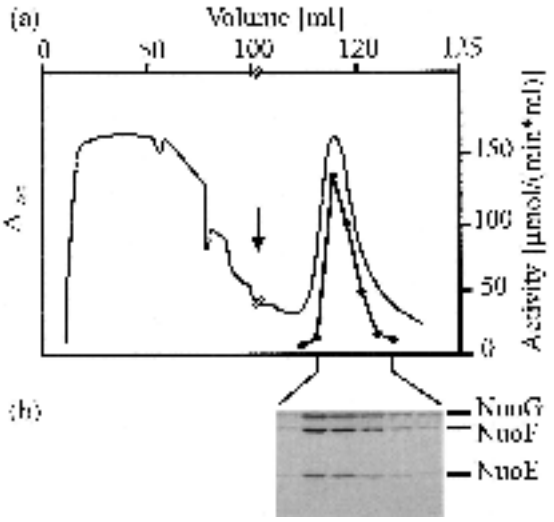

Fig. 2. a: Purification of the NADH dehydrogenase fragment fused with the *Strep*-tag II sequence C-terminal to NuoF by affinity chromatography on StrepTactin-Sepharose. Solid line, absorbance at 280 nm; ●-●, NADH/ferricyanide reductase activity. The arrow indicates the start of the elution with D-desthiobiotin buffer. b: SDS-PAGE of the fractions with NADH/ferricyanide activity. The proteins are assigned to the corresponding Nuo subunits.

plasmid pET-11a/*nuoB–G*/NuoF$_N$ overproduced the corresponding subunits as judged by SDS-PAGE (Fig. 1). The cytoplasm of these cells did not show an enhanced NADH/ferricyanide reductase activity nor the EPR signals typical for the FeS clusters of this fragment (data not shown). The cytoplasmic proteins were applied to a DEAE-ion exchange chromatography, but no protein exhibiting NADH/ferricyanide reductase activity eluted from this column (data not shown), indicating that the NADH dehydrogenase fragment was not assembled in these cells. SDS-PAGE of total cell lysates from the *E. coli* strains hosting plasmids pET-11a/*nuoB–G*/NuoE$_C$, pET-11a/*nuoB–G*/NuoF$_C$ or pET-11a/*nuoB–G*/NuoG$_C$ showed the overproduced subunits NuoB, CD, E, F and G and an increased NADH/ferricyanide reductase activity of the cytoplasm (Fig. 1 and Table 2). Thus, the fusion of the *Strep*-tag II sequence to the C-terminus of the Nuo proteins allowed the overproduction of the fully assembled NADH dehydrogenase fragment. The fragment containing the affinity peptide at the C-terminus of NuoE did not bind to the StrepTactin affinity material, most likely because the peptide is not presented at the surface of the fragment. The NADH dehydrogenase fragments with the *Strep*-tag II peptide C-terminal to NuoF or NuoG bound with high affinity to the StrepTactin column and were used for further analysis.

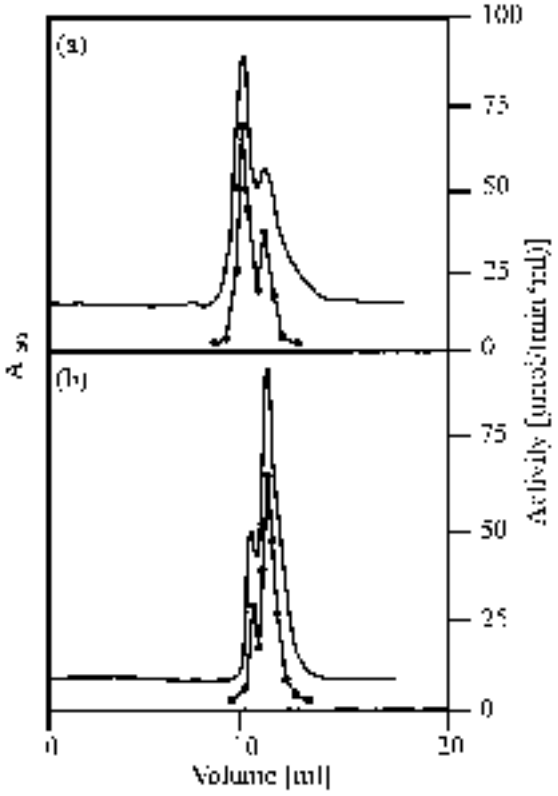

Fig. 3. Elution of the engineered NADH dehydrogenase fragment from size exclusion column Superdex 200 (1×30 cm, Pharmacia, Freiburg). Solid line, absorbance at 280 nm; ●-●, NADH/ferricyanide reductase activity. a: Chromatography in 50 mM MES-NaOH, 50 mM NaCl, pH 6.0. b: Chromatography in 50 mM MES-NaOH, 500 mM NaCl, pH 6.0.

3.2. Isolation of the tagged NADH dehydrogenase fragment by affinity chromatography

The tagged NADH dehydrogenase fragment eluted in a single peak from the affinity chromatography. SDS-PAGE revealed the presence of NuoE, F and G without any detectable impurities (Fig. 2). 15–20 mg of the fragment containing the affinity peptide fused to the C-terminus of NuoF was routinely obtained within 3 h (Table 3). The fragment containing the *Strep*-tag II peptide at the C-terminus of NuoG yielded 10–15 mg protein of the same purity.

Size exclusion chromatography resulted in two peaks of NADH/ferricyanide reductase activity corresponding to proteins with molecular masses of 340 and 170 kDa (Fig. 3). Both peaks show the same protein pattern in SDS-PAGE and the masses fit well with dimeric and monomeric form of the

Table 2
Influence of the position of the *Strep*-tag II sequence on production and assembly of the NADH dehydrogenase fragment and its binding to StrepTactin

E. coli strain BL21 (DE3) containing expression vector	Cytosolic NADH/ferricyanide reductase activity (μmol/min·mg)	Elution of a protein from ion exchange chromatography with NADH/ferricyanide reductase activity	Binding to StrepTactin
pET-11a	2.5	−	−
pET-11a/*nuoB–G*/NuoE$_N$	2.5	−	−
pET-11a/*nuoB–G*/NuoE$_C$	3.7	+	−
pET-11a/*nuoB–G*/NuoF$_N$	2.5	−	−
pET-11a/*nuoB–G*/NuoF$_C$	4.0	+	+
pET-11a/*nuoB–G*/NuoG$_C$	3.8	+	+

The indices N and C denote the position of the *Strep*-tag II sequence on the corresponding subunit.

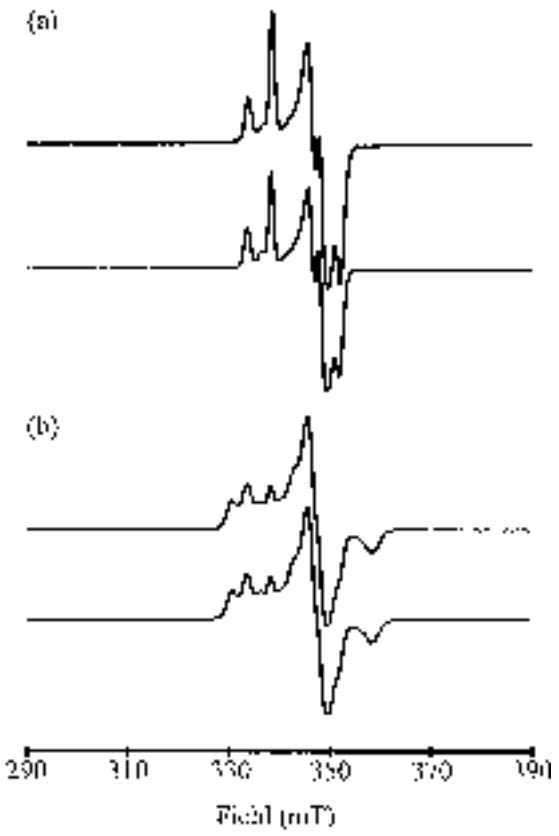

Fig. 4. EPR spectra of the isolated engineered NADH dehydrogenase fragment (upper curve) and the fragment obtained by standard chromatographic steps (lower curve) reduced with NADH were recorded at (a) 40 K and 2 mW microwave power and (b) 13 K and 5 mW microwave power. Other EPR conditions were: microwave frequency, 9.44 GHz; modulation amplitude, 6 mT; time constant, 0.064 s; scan rate, 17.9 mT/min.

NADH dehydrogenase fragment. The ratio of monomer to dimer is roughly 3:1 and could not be altered by incubating the preparation in 0.2% (w/v) dodecyl maltoside or 5 mM dithiothreitol (data not shown). Incubation in 50 mM MES-NaOH, 500 mM NaCl, pH 6.0 and subsequent chromatography in the same buffer reverses the ratio of monomer to dimer to 1:3 (Fig. 3). Therefore, the dimerization of the NADH dehydrogenase fragment is most likely caused by ionic interactions.

3.3. Properties of the tagged NADH dehydrogenase fragment

The purified NADH dehydrogenase fragment containing the affinity peptide C-terminal to either NuoF or NuoG shows a specific FMN content of 6.0–7.0 nmol/mg, and specific iron contents of 110–130 nmol/mg. Using a molecular mass of 170 kDa for the NADH dehydrogenase fragment as derived from the DNA sequence the preparation contains 1.0–1.2 mol FMN and 19–22 mol of iron per mol of NADH dehydrogenase fragment. Similar values have been obtained for the preparation of the untagged fragment [6]. Although two binuclear and two tetranuclear FeS clusters have so far been detected by EPR spectroscopy the presence of up to three binuclear and three tetranuclear clusters is predicted from sequence data [5]. The UV-Vis (data not shown) and EPR spectra (Fig. 4) of the tagged fragment are identical to the spectra of the untagged fragment [6].

4. Discussion

In order to establish a fast and gentle method to prepare the NADH dehydrogenase fragment of complex I, we fused the *Strep*-tag II affinity peptide to individual subunits of this fragment. This tag was selected due to the high binding affinity of the peptide to StrepTactin ($K_d = 10^{-6}$ M) and the mild conditions for elution of the bound protein [9]. Five constructs were made using PCR methods to fuse the peptide N-terminal to NuoE and NuoF and C-terminal to NuoE, NuoF or NuoG. These constructs were used to replace the corresponding wild type genes on the overexpression plasmid pET-11a/*nuoB–G* [6]. Fusion of the *Strep*-tag II coding sequence downstream of the start of *nuoE* prohibited the expression of the *nuo* genes (Fig. 1). Insertion of this sequence increases the distance between the ribosome binding site and the start of *nuoE* which is most likely the reason for the lack of expression. The proteins NuoB and NuoCD may not be stable without the other Nuo proteins and are most likely exposed to proteolytic degradation. The N-terminal fusion of the affinity peptide to NuoF led to overproduction of the individual proteins which were, however, not assembled (Fig. 1 and Table 2). The N-terminus of NuoF seems to interact with other subunits of the NADH dehydrogenase fragment, as has been observed for numerous multisubunit complexes [14]. Because of this we did not attempt to fuse the affinity peptide to the N-terminus of NuoG.

Engineering the *Strep*-tag II sequence to the C-termini of NuoE, F or G led to the overproduction of NuoB, CD, E, F and G and the assembly of NADH dehydrogenase fragment in the cytoplasm (Fig. 1 and Table 2). The fragment carrying the tag at NuoE did not interact with the StrepTactin affinity material while the C-terminal fusion to either NuoF or NuoG enabled us to purify the NADH dehydrogenase fragment using StrepTactin affinity chromatography (Fig. 3). The fragment was purified within 3 h yielding roughly 60% of the protein amount obtained from the isolation procedure using standard techniques [6]. However, the latter preparation took 2 days. Introduction of the *Strep*-tag II sequence to either NuoF or NuoG did not alter the spectroscopic or biochemical features of the overexpressed NADH dehydrogenase fragment.

To our knowledge, this is the first report of the purification

Table 3
Isolation of the overproduced and engineered NADH dehydrogenase fragment from the *E. coli* strain BL21 (DE3) pET-11a/*nuoB–G*/NuoF$_C$ from 30 g *E. coli* cells (wet mass)

Preparation	Volume (ml)	Protein (mg)	Specific NADH/ferricyanide reductase activity (μmol/min·mg)
Cytoplasm	75	1250	3.5
StrepTactin-Sepharose	9	18	113

The peak fractions were used.

of a multisubunit complex using direct fusion of the *Strep*-tag affinity peptide to a protein. For purification of the membranous, multisubunit cytochrome *c* oxidase from *Paracoccus denitrificans* the affinity peptide was fused to an engineered Fv fragment which bound to the epitope of the oxidase [15]. We are currently trying to use direct fusion of the affinity peptide for purification of the entire complex I.

Acknowledgements: We thank Monika Kerstan for her excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft.

References

[1] Walker, J.E. (1992) Q. Rev. Biophys. 25, 253–324.
[2] Friedrich, T. and Weiss, H. (1997) J. Theor. Biol. 187, 529–540.
[3] Brandt, U. (1997) Biochim. Biophys. Acta 1318, 79–91.
[4] Friedrich, T., Steinmüller, K. and Weiss, H. (1995) FEBS Lett. 367, 107–111.
[5] Weidner, U., Geier, S., Ptock, A., Friedrich, T., Leif, H. and Weiss, H. (1993) Mol. Biol. 233, 109–122.
[6] Braun, M., Bungert, S. and Friedrich, T. (1998) Biochemistry 37, 1861–1867.
[7] Friedrich, T. (1998) Biochim. Biophys. Acta 1364, 134–146.
[8] Leif, H., Sled, V.D., Ohnishi, T., Weiss, H. and Friedrich, T. (1995) Eur. J. Biochem. 230, 538–548.
[9] Schmidt, T.G.M. and Skerra, A. (1993) Protein Eng. 6, 109–122.
[10] Hanahan, D. (1983) J. Mol. Biol. 166, 557–565.
[11] Studier, F.W. and Moffat, B.A. (1986) J. Mol. Biol. 189, 113–130.
[12] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[13] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci USA 74, 5463–5467.
[14] Schulze, G.E. and Schirmer, R.H. (1979) Principles of Protein Structure, Springer Verlag, New York.
[15] Kleymann, G., Ostermeier, C., Ludwig, B., Skerra, A. and Michel, H. (1995) Bio/Technology 13, 155–160.