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EDDIE fusion proteins: Triggering autoproteolytic cleavage

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ABSTRACT

Heterologous proteins are often poorly expressed in *Escherichia coli* and especially small peptides are prone to degradation. N^{pro} autoprotease fusion proteins, deposited as inclusion bodies in *E. coli*, are a versatile tool for peptide and protein overexpression and generate an authentic N terminus at the target molecule. Autoproteolytic cleavage and subsequent release of the fusion partner are initiated upon refolding. Fusion proteins with the N^{pro} mutant EDDIE follow a monomolecular reaction. The reaction rate was only dependent on chaotrope concentration, decreasing exponentially by a factor of 1.2–1.5 for urea and by a factor of 2.1–5.3 for GuHCl. The first amino acid of the target peptide had a major impact on the reaction rate studying a set of model peptides. Reaction rates were in the range of 2.2×10^{-4} to 7.3×10^{-5} s^{-1} and could be increased up to fivefold by exchanging the first amino acid of the target peptide. A panel of biophysical methods was used to assess EDDIE secondary and tertiary structure. Immediate formation of secondary structure and slight increase in β-sheet content of approximately 5% over the course of the cleavage reaction was observed and interpreted as aggregation. Aggregation and cleavage occurred simultaneously. EDDIE has a relatively loose structure with the cleavage site exhibiting the lowest solvent exposure. We hypothesize that this is the mechanism for establishing a spatial proximity between cleavage site and the catalytic centre of the autoprotease. Fluorescence measurements revealed that further structural changes did not occur after the initial hydrophobic collapse. Thus, the overall reaction is predominantly controlled by cleavage kinetics and refolding kinetics does not play a major role.

1. Introduction

Recombinant proteins and peptides are often poorly expressed in *Escherichia coli* (*E. coli*), with small peptides being especially prone to intracellular degradation [1]. Therefore, several fusion tag strategies have been applied to enhance expression [2]. For proteins used for medical applications, not only is the expression yield important, but also the identity of the recombinant product with special respect to the N terminus. *E. coli* derived polypeptides are synthesised with formyl-methionine as the N-terminal amino acid [3]. Incomplete cleavage may lead to intolerable microheterogeneity of the product and even to changes in functionality and stability [4,5].

Previously, N^{pro} fusion technology has been introduced as a novel production platform for recombinant protein expression in *E. coli*. This technology makes use of the autoproteolytic function of N^{pro} derived from classical swine fever virus [6,7]. Most proteins and peptides fused to N^{pro} are deposited as inclusion bodies (IBs), which must be dissolved under chaotropic condition. Upon switching to cosmotropic refolding conditions, the autoprotease regains activity for autoproteolytic cleavage performed at its C terminus releasing the fused partner with an authentic N terminus. By using N^{pro} fusion technology exceptionally high titres can be achieved, even for toxic and poorly expressed proteins and peptides [8]. For preparative and industrial applications, a tailor-made N^{pro} mutant called EDDIE has been engineered (Table 1). EDDIE exhibits a better solubility as well as increased *in vivo* cleavage rates.

In a recent study [9], we showed that refolding and autoproteolytic cleavage of EDDIE fusion proteins comprising target proteins with slow refolding kinetics, e.g. GFP, do behave

Abbreviations: ACN, acetonitrile; ATR-FTIR, attenuated total reflectance Fourier transform infrared; *b*, k_0 associated parameter; *c*, chaotrope concentration; R^2, coefficient of determination; conc., concentration; C (Cys), cysteine; GuHCl, guanidine hydrochloride; 6His, hexahistidine; HCl, hydrochloride; IB, inclusion body; k_0, hypothetical maximum; k_1, overall rate constant of folding and cleavage; k_2, overall rate constant of misfolding; K (Lys), lysine; MTG, α-monothioglycerol; PLS-R, partial least squares regression; pep, peptide; RMSECV, root mean square error of cross-validation; RP, reversed phase; sSNEV, synthetic senescence evasion factor; *t*, time; TFA, trifluoroacetic acid; Tris, 2-amino-2-hydroxymethyl-propane-1,3-diol; *Y*, yield.

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Table 1
Primary sequence of EDDIE.

	1	11	21	31	41	51	
1	MELNHFELLY	KTSKQKPVGV	EEPVYDTAGR	PLFGNPSEVH	PQSTLKLPHD	RG**ED**DI**E**TTL	60
61	RDLPRKGDCR	SGNHLGPVSG	IYIKPGPVYY	QDYTGPVYHR	APLEFFDE**T**Q	F**EE**T**T**KRIGR	120
121	VTGSDGKLYH	IYV**E**VDG**E**IL	LK**Q**AKRGTPR	TLKW**T**RN**T**TN	CPLWVTSC		

Positions which had been exchanged with respect to the wild type N^{pro} are shown in bold.

according to the commonly applied refolding model of Kiefhaber et al. [10], where refolding follows a 1st order process and aggregation, a 2nd order reaction. According to the Kiefhaber model, aggregation is the major yield-limiting factor for protein refolding. In contrast, EDDIE fusion proteins comprising short polypeptides as targets showed refolding and cleavage yield independent of protein concentration. Moreover, the reaction rate was also constant over a wide protein concentration range up to 1.0 mg ml^{-1}. Based on these observations, we proposed a reaction scheme assuming a monomolecular reaction for the refolding and autoproteolytic cleavage process whereby a misfolding reaction follows 1st order kinetics:

$$Y(t) = \frac{k_1}{k_1 + k_2} - \frac{k_1}{k_1 + k_2} \cdot \exp(-(k_1 + k_2) \cdot t) \quad (1)$$

with $Y(t)$ being the reaction yield as the quotient of folded (and cleaved) protein and starting protein concentration, k_1 (s^{-1}) the overall rate constant of folding and cleavage, k_2 (s^{-1}) the overall rate constant of misfolding, and t (s) the time. However, for protein concentrations higher than 1.0 mg ml^{-1}, the proposed model failed to properly describe the determined kinetics. This discrepancy could only partially be explained by residual urea required for dissolution of IBs in the refolding buffer. Furthermore, the fusion partner strongly influenced the refolding and cleavage kinetics of the EDDIE fusion proteins. Comparing two peptides, reaction rates differed by more than one order of magnitude.

In the present study, we conducted a detailed examination of the impact of type and concentration of chaotrope and the influence of the sequence of EDDIE fusion partners on refolding and autoproteolytic cleavage of EDDIE fusion proteins. We conducted experiments with EDDIE fused to a short sixteen amino acid peptide (pep6His) consisting of a 10 amino acid spacer with a C-terminal polyhistidine tag. The sequence of the spacer was derived from the multiple cloning site of the pET30a vector used for overexpression of fusion proteins. The polyhistidine tag was introduced to ease purification and detection. Another peptide investigated was the inhibitorial peptide of senescence evasion factor (sSNEVi-C) [11]. In order to investigate the impact of the amino acid composition of the target peptide, variants of the fusion partners were designed with the first N-terminal amino acid being exchanged.

The present work also focused on the correlation between batch refolding characteristics and cleavage kinetics of EDDIE, a novel protein of unknown secondary and tertiary structure. We sought a clearer understanding of the native conformation of EDDIE and of the role of EDDIE conformation changes in the autoproteolytic cleavage reaction. Initial protein folding events can occur very rapidly [12,13]. The initial hydrophobic collapse and the assembly of secondary structural elements take place in the micro- to millisecond range. Fast folding proteins even gain their native conformation in this time frame. For us, the time frame which could be accessed experimentally was between 1 and 1000 min, the time range in which refolding and cleavage kinetics were observed. The important question in this context was to elucidate whether refolding and cleavage are two independent, sequentially occurring reactions or if they occur in parallel. Additionally, it was of interest if refolding kinetics and cleavage kinetics are interconnected. Therefore, we applied a panel of biophysical methods to follow possible structural changes during the entire refolding and cleavage process.

Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy is a method frequently used for protein secondary structure analysis [14,15]. Turbidity measurements, respectively light scattering are standard techniques to study protein aggregation [16–19]. Time-resolved fluorescence studies [20], and fluorescence quenching to analyze protein structure [21] and folding as well as unfolding kinetics [22,23] are frequently applied methods in protein biochemistry. Both, tryptophan [24,25] and tyrosine [26,27] are potential targets for quenching studies.

Herein, we report our observations with the aforementioned analytical methods of secondary and tertiary structure as well as aggregation analysis and interpret their outcomes in the context of the observed refolding and cleavage kinetics of various EDDIE fusion proteins. Using these data, we have attempted to establish a better understanding of the EDDIE cleavage mechanism on a molecular level.

2. Materials and methods

2.1. Protein and buffer preparation

The recombinant fusion proteins EDDIE-pep6His, EDDIE-K-pep6His, EDDIE-sSNEVi-C and EDDIE-C-sSNEVi were overexpressed in *E. coli* strain BL21 (DE3) with a pET30a plasmid (Novagen, Madison, WI, USA) containing the respective coding gene [8]. *E. coli* fed batch cultivations were conducted with semi-synthetic medium on a 5 l scale according to Clementschitsch et al. [28]. IBs were harvested by centrifugation, followed by a number of washing steps with water and detergents. Details have been described by Kaar et al. [9].

IBs were dissolved in a buffer containing 10.0 M urea, 50 mM Tris, and 100 mM MTG at pH 7.3. Urea and Tris were purchased from Merck (Darmstadt, Germany), MTG from Sigma–Aldrich (Vienna, Austria). The dissolved IBs were then centrifuged for 30 min at 16,110 × *g* and 277 K. Insoluble components were removed by subsequent filtration through 0.4 and 0.22 μm filters (Millipore, Billerica, USA). Protein concentration was determined with a Cary 50 Bio UV–vis spectrophotometer (Varian, Palo Alto, USA) at 280 nm.

2.2. Protein refolding

Protein refolding was performed as previously described [9]. Refolding of the IB protein was initiated by rapid dilution into refolding buffer containing 1 M Tris, 0.25 M D(+)-sucrose, 2 mM EDTA, 20 mM MTG at pH 7.3 and residual levels of chaotropic agents from dissolution buffer. D(+)-Sucrose was purchased from Acros Organics (New Jersey, USA). Since the protein concentration for refolding experiments was determined by adding the solution of dissolved IB into the refolding buffer, the amount of residual chaotropic reagent increased with increasing protein concentration. Exceptions were experiments investigating the influence of type and concentration of chaotrope, where refolding buffers with defined chaotrope concentrations independent of the protein concentration were used. Refolding samples were incubated at 291 K without further stirring. Immediately after transfer of protein to refolding buffer, an aliquot of the refolding sample was subjected to RP-HPLC analysis, performed at distinct time intervals from the same vial to follow the time course of refolding.

2.3. RP-HPLC analysis

Protein analyses by RP-HPLC were performed with a Jupiter™ C-4 column (2 mm × 150 mm, 5 μm, 300 Å) (Phenomenex, Torrance, CA, USA) on Agilent 1100 series chromatographic system (Waldbronn, Germany) using an additional SecurityGuard™-cartridge. A buffer system of 0.1% (v/v) TFA, 5% (v/v) ACN