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Catalytically-active inclusion bodies—Carrier-free protein immobilizates for application in biotechnology and biomedicine

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ABSTRACT

Bacterial inclusion bodies (IBs) consist of unfolded protein aggregates and represent inactive waste products often accumulating during heterologous overexpression of recombinant genes in *Escherichia coli*. This general misconception has been challenged in recent years by the discovery that IBs, apart from misfolded polypeptides, can also contain substantial amounts of active and thus correctly or native-like folded protein. The corresponding catalytically-active inclusion bodies (CatIBs) can be regarded as a biologically-active sub-micrometer sized biomaterial or naturally-produced carrier-free protein immobilizate. Fusion of polypeptide (protein) tags can induce CatIB formation paving the way towards the wider application of CatIBs in synthetic chemistry, biocatalysis and biomedicine. In the present review we summarize the history of CatIBs, present the molecular-biological tools that are available to induce CatIB formation, and highlight potential lines of application. In the second part findings regarding the formation, architecture, and structure of (Cat)IBs are summarized. Finally, an overview is presented about the available bioinformatic tools that potentially allow for the prediction of aggregation and thus (Cat)IB formation. This review aims at demonstrating the potential of CatIBs for biotechnology and hopefully contributes to a wider acceptance of this promising, yet not widely utilized, protein preparation.

1. Introduction

The use of enzymes has become increasingly popular in both industry and lab-scale synthetic chemistry, mainly due to the fact that they catalyse certain chemical reactions with unprecedented efficacy, specificity, and stereoselectivity, which is often not attainable employing conventional chemical catalysts (Nestl et al., 2011; Patel et al., 2003). However, industrial processes often require harsh conditions such as extreme pH values, high temperatures or the use of organic solvents (Castro and Knubovets, 2003). In particular the use of organic solvents, enabling high substrate and product concentrations (i.e. for dissolving hydrophobic substrates and products), represents a promising avenue to intensify a given process i.e. in terms of space-time-yield (STY) and thus economic gain. While it has been shown in the recent past that many enzymes can maintain their activity in non-aqueous reaction media such as organic solvents, ionic liquids, and supercritical fluids (Castro and Knubovets, 2003; Khmelnitsky and Rich, 1999; Sheldon, 2016), those conditions nevertheless set far higher requirements on the stability of the employed enzyme. Therefore, enzymes are often immobilized in/or on carrier materials, resulting in improved catalytic performance with regard to stability, handling and recycling (Balcao and Vila, 2014; Guisan, 2013; Liese and Hilterhaus, 2013; Sheldon, 2011; Tielmann et al., 2014; Varalakshmi and Siva rama prasad, 2013). Although a broad range of methods is now available for enzyme immobilization (recently reviewed in (Es et al., 2015; Guisan, 2013)), no generic concepts are available and enzyme immobilization has to be optimized on a case to case basis. Likewise, the purification and production of large amounts of enzyme is often laborious and thus cost intensive, which hampers the application of enzymes, e.g. for the industrial production of bulk chemicals (Tufvesson et al., 2011). Therefore, generic methods that enable the production of large amounts of highly pure, active, and stable enzyme immobilizates are urgently needed. Ideally, those immobilizates should be obtainable in pure form without the need for chromatographic purification and/or additional immobilization steps. Recently more and more evidence has been provided that enzymes/proteins can retain their biological activity under certain conditions when produced as insoluble inclusion bodies (IBs) (Arie et al., 2006; Diener et al., 2016; Garcia-Fruitos et al., 2005;

Abbreviations: CatIBs, catalytically-active inclusion bodies

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Korovashkina et al., 2013; Lin et al., 2013; Nahalka, 2008; Nahalka et al., 2006; Nahalka and Nidetzky, 2007; Nahalka and Patoprsty, 2009; Nahalka et al., 2008; Park et al., 2012; Wang et al., 2015; Worrall and Goss, 1989; Wu et al., 2011). Those catalytically-active IBs (CatIBs) essentially represent a biologically produced enzyme immobilizate, whose use can circumvent many of the aforementioned problems. IBs are intracellularly formed insoluble protein aggregates that have long been assumed to be unfolded or misfolded and thus inactive waste, produced during heterologous gene expression (Baneyx and Mujacic, 2004). Within this review we will use the term CatIBs for IBs that possess biological activity, referring either to the catalytic activity of enzymes, the fluorescence of fluorescent reporter proteins or other biological activities (e.g. for therapeutic proteins that do not *per se* possess catalytic activity). To account for the fact that the abbreviation CatIBs, which stands for catalytically-active IBs is misleading in the latter two cases, we use in such an instances (Cat)IBs (Cat in parentheses). The term IBs is used for "conventional" inactive IBs. Traditionally, IBs are often discarded from further characterization or are at most used for refolding studies to obtain soluble protein (Singh et al., 2015). However, a careful literature survey reveals a few anecdotal examples that describe CatIBs for naturally occurring proteins (Dong et al., 2014; Garcia-Fruitos et al., 2005; Li et al., 2013; Nahalka, 2008; Nahalka and Patoprsty, 2009; Nahalka et al., 2008; Park et al., 2012; Tokatlidis et al., 1991; Worrall and Goss, 1989). More importantly, recent studies have shown that the formation of CatIBs can be induced by the fusion of a variety of different aggregation inducing tags or domains (Arie et al., 2006; Choi et al., 2011; Diener et al., 2016; Garcia-Fruitos et al., 2005; Lin et al., 2013; Nahalka and Nidetzky, 2007; Park et al., 2012; Peternel and Komel, 2011; Rehm et al., 2016; Wang et al., 2015; Wu et al., 2011; Zhou et al., 2012). CatIBs nowadays represent a promising but as yet underutilized source of enzyme material for biotechnological applications that can be expected to gain increasing use in both industry and lab-scale synthetic chemistry in the near future. Here, we present a brief history of CatIBs and describe potential lines of application. Recent findings regarding the formation, architecture and structure of IBs are presented which may allow the more rational design of (fusion) proteins for CatIB formation in the future.

2. A brief history of (Cat)IBs: naturally occurring protein IBs with catalytic activity

A careful survey of the literature reveals that catalytically-active IBs (CatIBs) of naturally occurring proteins have been reported as early as 1989, where Worrall and Goss demonstrated that the enzyme β-galactosidase can be produced as CatIBs in *Escherichia coli* (Worrall and Goss, 1989). In 1991, Tokatlidis and co-workers demonstrated that the endoglucanase D of *Clostrium thermocellum* is likewise produced as CatIBs (Tokatlidis et al., 1991). Other more recent examples of native proteins that yield CatIBs in *E. coli* are a human dihydrofolate reductase (Garcia-Fruitos et al., 2005), the pyruvate oxidase of *Paenibacillus polymyxa* (Park et al., 2012), a lipase from *Serratia marcescens* (Li et al., 2013), a β-galactosidase from *Pyrococcus furiosus* (Dong et al., 2014) as well as a polyphosphate kinase from *E. coli* (Nahalka et al., 2006) a diguanylate cyclase from *Thermatoga maritima* (Korovashkina et al., 2013), and a loop-deletion variant of the green-fluorescent protein (GFP) (Raghunathan et al., 2014; Raghunathan et al., 2012). Further details about the respective CatIBs, including their specific activities, origin, expression host strains and enzyme class are summarized in Table 1.

The presented literature summary demonstrates that IBs of recombinant proteins can contain substantial amounts of catalytically-active, and thus most likely correctly folded, protein. Nonetheless, in most present studies in which IBs are encountered upon overexpression of a target gene, they are still rarely tested for activity. At the same time, there are clear examples for IBs lacking catalytic activity (Chen et al., 2003; Joerger and Haas, 1993; Ren et al., 2000; Upadhyay et al., 2014). Thus, it is presently not possible to judge if (catalytic) activity is an intrinsic feature of all IBs (at least to some extend) or only a feature of certain specific target proteins. The diverse characteristics of naturally CatIB forming proteins, e.g. different enzyme classes, different structure and origin, would argue against this notion. The literature research is further complicated by the fact, that in many cases catalytic activity of recombinant IB-forming proteins was simply not measured (Garcia-Fruitos, 2010). More likely, the tendency of a given protein to form CatIBs is determined by yet undetermined sequence/structural features in combination with specific expression conditions (see also chapter 5-7 below). It should be noted that the collection of CatIBs from naturally-occurring proteins shown in Table 1 is probably by no means complete, as corresponding reports are inherently difficult to find in literature databases among the vast numbers of papers dealing with enzyme IBs and refolding studies.

3. Engineering of CatIB formation by fusion protein generation

In recent years, the formation of CatIBs has been achieved by molecular biological fusion of a variety of structurally different polypeptide and protein tags to various structurally and functionally dissimilar target proteins and enzymes (Table 2; see references therein). Alternative methods relying on related principles such as inverse thermal cycling (ITC) enabled by the fusion of elastine-like peptides, extended variants of the green fluorescent protein GFP (Venning-Slater et al., 2014), crosslinked enzyme aggregate systems as well as silica and polyhydroxyalkanoate (PHA) binding proteins have recently been reviewed (Roessl et al., 2010) and will not be covered here.

3.1. Artificial self-assembling peptides

A large group of CatIB-formation inducing tags is constituted by small artificial self-assembling peptides. They vary in length between 8 and 18 amino acids and have been fused to the C-terminus of the respective target in all presented examples. The first reported example of CatIB-formation inducing peptides was the amphipathic ELK16 peptide (Wu et al., 2011), a more hydrophobic version of the self-assembling β-peptide EAK16, which originates from the sequence of the Z-DNA binding protein zuotin (Zhang et al., 1992). The corresponding enzyme CatIBs (demonstrated for *Aspergillus fumigatus* amadoriase II and a xylosidase of *Bacillus pumilus*) retained a high degree of specific activity compared to the native (soluble) enzyme. Moreover, Fourier Transform Infrared (FT-IR) spectroscopic evidence was provided for an increased formation of extended antiparallel pleated β-sheet structures in the corresponding CatIBs that are indicative for amyloid-like structural properties of the aggregates (Wu et al., 2011) (see also chapter 6 below). In a later study, different small surfactant-like peptide sequences, which were designed based on similarity to previously reported small surfactant-like peptides that self-assemble to form nanotubes and nanovesicles (Vauthey et al., 2002; von Maltzahn et al., 2003), were fused to different target proteins to induce CatIB formation (Zhou et al., 2012). Similar to surfactant molecules, surfactant-like peptides contain a hydrophobic tail and a hydrophilic head. One of the best studied tags of this category is the 8 amino acids long L_6KD peptide (Zhou et al., 2012), which was used to induce CatIB formation for three different enzymes (*Bacillus subtilis* lipase A, *Aspergillus fumigatus* amadoriase II and a xylosidase of *Bacillus pumilus*) and the fluorescent reporter GFP (green fluorescent protein) (Zhou et al., 2012). Additional to L_6KD, two other variants of the tag (L_6K_2, DKL_6) were fused to the *A. fumigatus* amadoriase II gene. All three peptide variants induced the formation of amadoriase II CatIBs to a variable extend (Zhou et al., 2012). Interestingly, proteinase K digested CatIBs of one of the fusion proteins revealed fibril-like structures and bound the amyloid-specific dye thioflavin-T (Th-T), indicating that the corresponding aggregates exhibit amyloid-like structures (Zhou et al.,