Reversible Inhibition of Protein Splicing by Zinc Ion*

Received for publication, December 11, 2000, and in revised form, January 10, 2001
Published, JBC Papers in Press, January 10, 2001, DOI 10.1074/jbc.M011149200

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Protein splicing involves the self-catalyzed excision of a protein-splicing element, the intein, from flanking polypeptides, the exteins, which are concomitantly joined by a peptide bond. Taking advantage of recently developed in vitro systems in which protein splicing occurs in trans to assay for protein-splicing inhibitors, we discovered that low concentrations of Zn\(^{2+}\) inhibited splicing mediated both by the RecA intein from Mycobacterium tuberculosis and by the naturally split DnaE intein from Synechocystis sp. PCC6803. Inhibition by Zn\(^{2+}\) was also observed with a cis-splicing system involving the RecA intein. In all experimental systems used, inhibition by Zn\(^{2+}\) could be completely reversed by the addition of EDTA. Zinc ion also inhibited hydroxylamine-dependent N-terminal cleavage of the RecA intein. All other divalent transition metal ions tested were less effective as inhibitors than Zn\(^{2+}\). The reversible inhibition by Zn\(^{2+}\) should be useful in studies of the mechanism of protein splicing and allow structural studies of unmodified protein-splicing precursors.

Protein splicing involves the excision of an intervening polypeptide sequence, the intein, from a precursor protein and the concomitant joining of the flanking polypeptides, the exteins, by a peptide bond (see Ref. 1 for a recent review of protein splicing). The in vitro biochemical study of protein splicing has been hampered by the fact that it is a self-catalyzed process that requires neither accessory proteins nor cofactors (2) and therefore proceeds rapidly under physiological conditions without the accumulation of intermediates. However, recent progress in the molecular dissection of inteins has made possible the expression of intein segments as fusion proteins that undergo protein splicing in trans after reconstitution in vitro (3, 4). This advance as well as the recent discovery of a naturally occurring trans-splicing system (5, 6) has opened the way for the in vitro characterization of the protein-splicing process.

Protein splicing in trans requires the reassociation of an N-terminal and C-terminal fragment of an intein (N-intein and C-intein, respectively), each fused to an appropriate extein. Upon reassociation, the intein fragments form a functional protein-splicing active center, which mediates the formation of a peptide bond between the exteins, coupled to the excision of the N- and C-inteins (Fig. 1). In the experiments described in this paper, we used two different in vitro trans-splicing systems. The first was derived from the Mycobacterium tuberculosis RecA intein. It consisted of a 105-residue N-intein fused to Escherichia coli maltose-binding protein (MBP)§ as the N-extein and a 107-residue C-intein fused to a polypeptide terminated with a His tag as the C-extein (4). The functional reassociation of the RecA intein segments requires prior denaturation and joint renaturation (4), even if the C-intein is replaced by a polypeptide as short as 35 amino acid residues (7). The second trans-splicing system was based on the naturally split DnaE intein of Synechocystis sp. PCC6803 (8), which has a 123-residue N-intein and a 36-residue C-intein and can undergo reassociation without prior denaturation (6). For the purpose of these studies, we fused the DnaE N-intein, joined to the 16 C-terminal residues of the natural C-extein, to E. coli MBP and a synthetic C-extein fused to the 5 N-terminal residues of the natural C-extein, followed by a His tag.

A recent crystallographic study of a Saccharomyces cerevisiae VMA intein analog fused to short extein segments revealed that the intein contains a bound zinc ion (9). We decided to explore the effects of zinc ion on protein splicing using the experimental systems described above and discovered that zinc ion acts as a general and reversible inhibitor of protein splicing. No inhibitor of protein splicing had been available until now, and the reversible inhibition by Zn\(^{2+}\) therefore provides a val-

* The abbreviations used are: MBP, E. coli maltose-binding protein; Bis-Tris-Propane, 1,3-bis-([tris(hydroxymethyl)methylamino] propane); TCEP, tris(2-carboxyethyl) phosphine; His tag, hexahistidine sequence; PAGE, polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption ionization/time of flight spectroscopy; H, a 49-amino acid peptide with a C-terminal His tag; H'; the undecapeptide MHHHHHHHHPLSG; M, N-terminal extein containing a spacer and N-terminal MBP; U\(_{\text{Na}}\), the 105 N-terminal amino acids of the M. tuberculosis RecA intein followed by the sequence CA; S N\(_{\text{A35}}\), chimeric protein consisting of M joined to the 16 C-terminal residues of the natural C-extein, followed by a His tag.

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§ The work was supported by National Institutes of Health Grant R01 GM55875 (NIGMS) (to H. P.); and by a Howard Hughes Medical Institute predoctoral fellowship (to K. V. M.). The mass spectrometer used in this work was funded by National Institutes of Health Grant RR11301 and National Science Foundation Grant 96-04781. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ The sequence of the DnaE intein segment is represented schematically in Fig. 2.
Ubiquitous tool for the study of the mechanism of protein splicing and intein structure.

**EXPERIMENTAL PROCEDURES**

**Plasmid Preparation**—Plasmid pMSN encodes MSN, an in-frame fusion of the E. coli MBP with the 12 C-terminal intein residues and the 123 intein residues of the N-terminal DnaE fragment of Synechocystis sp. PCC6803. The corresponding segment of the N-terminal dhaE fragment was amplified by polymerase chain reaction from genomic DNA, which had been isolated from Synechocystis sp. PCC6803 (obtained from the American Type Culture Collection as strain 27184) as described (10) and used to replace the six C-terminal residues of MBP and the entire RecA intein in plasmid pMU2 s/sΔ6 (4). Plasmid pMSN can thus be considered a derivative of plasmid pMal-c2x (New England Biolabs) encoding a fusion protein in which amino acid 387 of MBP is fused to amino acid 759 of the Ssp DnaE N-terminal fragment. Plasmid pHU encodes H, which consists of the N-terminal sequence HHHHHHHPLSG (H9), fused in-frame to the 222-residue RecA mini-intein, MHHHHHHHPLSG (H), fused by a 49-amino acid polypeptide with a C-terminal His tag, described earlier (4). Plasmid pHU encodes H, which consists of the N-terminal extein, MHHHHHHHPLSG (H), fused by a 49-amino acid polypeptide with a C-terminal His tag (11). It was derived from pMU5H by replacing the coding sequence for MBP with a synthetic oligonucleotide encoding MHHHHHHHPLSG. Plasmid pMU5H differs from pMU5H by mutations that led to the replacement of the Asn-Cys sequence at the C-terminal splice junction by Ala-Ala, yielding the fusion protein MU*H. The intein fusion proteins encoded by these plasmids are represented schematically in Fig. 2.

**Peptide Synthesis**—Peptides were synthesized and purified as described (7). The 47-residue peptide SΔ35-A35 consists of the 36 residues of the Synechocystis DnaE C-terminal intein, followed by the sequence CFNKSHHHHHH. Peptide SΔ35-A35-H35 is identical to SΔ35-A35, except that it contains histidine at position 35 instead of alanine. Peptide U35 consists of the 38 C-terminal residues of the...
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Proteins derived from the M. tuberculosis RecA intein

| Peptide | Sequence |
|---------|----------|
| MU_{\text{H}} | MBP \cdots C \cdots U_{\text{L}} \cdots H_{\text{N}} |
| MU_{\text{N}} | MBP \cdots C \cdots U_{\text{N}} |
| U_{\text{C}} | MBP \cdots C \cdots U_{\text{A}} \cdots H_{\text{N}} |
| U_{\text{C36}} | MBP \cdots C \cdots U_{\text{A}} \cdots H_{\text{N}} |
| H'_{\text{U}} | MBP \cdots C \cdots U_{\text{A}} \cdots H_{\text{N}} |
| MU'_{\text{A}} | MBP \cdots C \cdots U_{\text{A}} \cdots H_{\text{N}} |

Proteins derived from the Synechocystis DnaE intein

| Peptide | Sequence |
|---------|----------|
| MS_{\text{N}} | MBP \cdots C \cdots S_{\text{N}} |
| S_{\text{C}} H'^{-} A35 | MBP \cdots C \cdots S_{\text{C}} \cdots ANCFNKSH_{\text{5}} |
| S_{\text{C}} H'^{-} H35 | MBP \cdots C \cdots S_{\text{C}} \cdots H_{\text{N}} |

**Fig. 2.** Schematic representation of the protein constructs used in this work. The diagram shows the key residues at the C-terminal splice junction and their mutations. The names of the constructs are derived as described in the abbreviation footnote. The extein segments are not shown to scale.

**M. tuberculosis** RecA intein followed by the sequence CA. Before use, the peptides were dissolved in buffer A, and their concentrations were estimated on the basis of their cysteine content by the method of Ellman.

**Reassociation and Protein-splicing Conditions**—Protein splicing and hydroxylamine-induced cleavage mediated in trans by the RecA intein were studied by mixing 30 \( \mu \text{M} \) MU_{\text{N}} and 60 \( \mu \text{M} \) U_{\text{C}} in 180 \( \mu \text{l} \) of buffer C supplemented with 8 mM urea or by mixing 20 \( \mu \text{M} \) MU_{\text{N}} and 30 \( \mu \text{M} \) U_{\text{C36}} CA in 300 \( \mu \text{l} \) of buffer C followed by dialysis at 4 \( ^\circ \text{C} \) for sequential 20-min periods against 50 \( \text{ml} \) each of the following buffers: buffer C supplemented with 8, 4, 2, 1, 0.5 M urea and no urea followed by overnight dialysis at 4 \( ^\circ \text{C} \) against buffer C. All dialyses were done in the presence of 1 \( \text{mM} \) tris(2-carboxyethyl)phosphine (TCEP) and in some cases in the presence of ZnCl\(_2\). Protein splicing was allowed to proceed at 25 \( ^\circ \text{C} \) for further addition of 1 \( \text{mM} \) TCEP and in some cases in the presence of 10 mM EDTA or 2.0 mM ZnCl\(_2\). Hydroxylamine-induced cleavage was studied by incubation at 25 \( ^\circ \text{C} \) with 1 \( \text{mM} \) TCEP and 0.5 M hydroxylamine, pH 7.0, and, when appropriate, with 10 \( \text{mM} \) EDTA. Splicing of H'_{\text{U}} was studied in a similar manner at a protein concentration of 3 \( \mu \text{g} \) per 100 \( \mu \text{l} \) reaction mixture and the sample was incubated for 30 min at 25 \( ^\circ \text{C} \) and then dialyzed against three changes of 30 mM urea.

**RESULTS**

**Effect of Zn\(^{2+}\) on Protein Splicing in Trans by RecA Intein Fragments**—In the search for possible inhibitors of protein splicing, we observed inhibition of protein splicing when ZnCl\(_2\) was present during the reconstitution of the RecA intein fragments and the subsequent splicing of the fusion proteins, MU_{\text{N}} and U_{\text{C}}. The yield of spliced protein (MH) after 16 h at 4 \( ^\circ \text{C} \) in the absence of zinc was 64% but was reduced to 1% in the presence of 2 \( \text{mM} \) ZnCl\(_2\) (Fig. 3, lanes 1 and 2). The inhibition by ZnCl\(_2\) was almost completely reversed by incubation at 25 \( ^\circ \text{C} \) for 2.5 h in the presence of 10 \( \text{mM} \) EDTA, whereas a similar incubation without EDTA had little effect (Fig. 3, lanes 3 and 4). Under similar conditions, 0.2 \( \text{mM} \) ZnCl\(_2\) inhibited protein splicing by 90%. In another experiment, the peptide U_{\text{C36}} CA, which does not contain a His-tag domain, was used in place of U_{\text{C}} in the splicing experiment. In this case, the yield of spliced product (M-Cys-Ala) was 53% after reassociation at 4 \( ^\circ \text{C} \) for 16 h in the absence of zinc ion but only 11% in the presence of 0.2 \( \text{mM} \) ZnCl\(_2\).

To determine whether ZnCl\(_2\) also inhibits protein splicing when added after the reassociation of the intein fragments, we reconstituted MU_{\text{N}} and U_{\text{C}} as above but in the absence of both TCEP and ZnCl\(_2\). After reassociation at 4 \( ^\circ \text{C} \) for 16 h, no discernable spliced product was observed. Upon incubation at 25 \( ^\circ \text{C} \) for 2.5 h in the presence of 1 \( \text{mM} \) TCEP, 48% splicing was observed in the absence of Zn\(^{2+}\) but only 5% in the presence of 2 \( \text{mM} \) ZnCl\(_2\).

To examine the ability of the Zn\(^{2+}\)-inhibited intein to catalyze partial reactions such as hydroxylamine-dependent N-terminal cleavage, we reconstituted the trans-splicing components in the presence of 2 \( \text{mM} \) ZnCl\(_2\) and then incubated for 2.5 h at 25 \( ^\circ \text{C} \) with ZnCl\(_2\) and 0.5 M hydroxylamine, pH 7.0. N-terminal cleavage and protein splicing were almost completely suppressed, but the chelation of Zn\(^{2+}\) by the addition of 10 \( \text{mM} \) EDTA in a parallel incubation restored N-terminal cleavage to 54%, accompanied by 16% splicing (Fig. 3, lanes 5 and 6).

**Effect of Zn\(^{2+}\) on cis-Protein Splicing and N-terminal Cleavage of a RecA Mini-intein**—Generally, cis-protein splicing cannot be studied in vitro on account of extensive splicing of the precursor protein during in vivo expression. However, H'_{\text{U}}H, a fusion protein in which a 222-residue RecA mini-intein (U_{\text{A}}) (11) was inserted between two His-tagged polypeptides (H' and...
H), was expressed in the insoluble fraction as unspliced precursor. After solubilization in 8 M urea, purification on a Talon column, and renaturation under reducing conditions, H’U₉H underwent extensive protein splicing. Analysis by SDS-PAGE indicated 73% protein splicing upon renaturation followed by incubation at 25 °C for 2.5 h in the absence of ZnCl₂ but no splicing in the presence of 2 mM ZnCl₂. Unfortunately, owing to the small size of the exteins, SDS-PAGE could not clearly distinguish between protein splicing and partial reactions such as cleavage at the splice junctions, which would yield products of a similar size. Accordingly, we used MALDI-TOF mass spectrometry for the definitive identification of the reaction products. Analysis of H’U₉H after renaturation and incubation in the presence of 2 mM ZnCl₂ followed by treatment with iodoacetamide to prevent protein splicing during sample preparation for mass spectrometry showed primarily a 30.7-kDa component (Fig. 4A), corresponding in mass to the acetamido derivative of the precursor H’U₉H (expected M, 30.8). When H’U₉H was refolded in the presence of ZnCl₂ and then incubated for 2.5 h at 25 °C in the presence of 10 mM EDTA, the 30-kDa component was much reduced and replaced by a 24.1-kDa species (Fig. 4B), whose size was consistent with that of the excised intein, U₉ (expected M, 24.1). Both samples also contained small amounts of a 25-kDa component, which could be the product of C-terminal cleavage, H’U₉ (expected M, 25.4).

To determine whether zinc ion can exert inhibitory effects on a fully folded intein, we studied the inhibition by zinc of hydroxylamine-dependent N-terminal cleavage of MU₁₉₉H. MU₁₉₉H contains U₉, an intein in which the second and third steps of protein splicing, transesterification and asparagine cyclization, are blocked by replacing the residues Asn-Cys at the C-terminal splice junction with Ala-Ala. MU₁₉₉H was purified under native conditions by affinity chromatography (Fig. 5, lane 1) and then incubated with 0.5 M hydroxylamine, pH 7.0, in the absence (lane 2) or presence (lane 3) of 2 mM ZnCl₂. The samples were then analyzed by SDS-PAGE together with molecular mass standards.

In Vitro trans-Splicing System Based on the Naturally Split DnaE Intein from Synechocystis sp. PCC6803 and the Role of the Penultimate C-Intein Residue in Protein Splicing—In contrast to the fragments derived from the M. tuberculosis RecA intein, whose functional reconstitution requires prior denaturation and renaturation, the naturally split DnaE intein from Synechocystis sp. PCC6803 reconstitutes without the need for prior unfolding (6). An interesting aspect of the Synechocystis sp. PCC6803 DnaE intein is that the canonical His adjacent to the intein C terminus, which is a potential metal ligand (9), is replaced by Ala (8). Although in vitro protein splicing was previously observed both with the wild-type (Ala-35) C-terminal intein segment (6) and with a mutant (His-35) C-terminal intein segment in which Ala-35 was replaced by His (14), these studies were done in very different contexts, and it is therefore

![Fig. 3. Reversible inhibition by zinc ion of trans splicing and of hydroxylamine-induced N-terminal cleavage mediated by RecA intein fragments. MU₁₉₉ and U₉ were renatured as described under “Experimental Procedures” and allowed to undergo protein splicing at 4 °C either in the absence (lane 1) or presence (lane 2) of 2 mM ZnCl₂. A portion of the sample analyzed in lane 2 was then incubated for 2.5 h at 25 °C with an additional 1 mM TCEP and either with (lane 3) or without (lane 4) 10 mM EDTA. Another portion of the sample from lane 2 was supplemented with an additional 1 mM TCEP and 0.5 M hydroxylamine, pH 7.0, and was then incubated for 2.5 h at 25 °C with (lane 5) or without (lane 6) 10 mM EDTA. The samples were then analyzed by SDS-PAGE together with molecular mass standards.](http://www.jbc.org/)

![Fig. 4. Mass spectrometric analysis of reversible inhibition of cis splicing by zinc ion mediated by a renatured RecA mini-intein. H’U₉H was renatured in the presence of 2.0 mM ZnCl₂ as described under “Experimental Procedures.” An aliquot was treated with iodoacetamide to prevent protein splicing during processing and then analyzed by MALDI-TOF mass spectrometry (A); another aliquot was allowed to incubate at 25 °C for 2.5 h with 10 mM EDTA and then analyzed by MALDI-TOF mass spectrometry (B). The mass numbers of the peaks are indicated in the figure, and the deduced corresponding molecular species are indicated in parentheses. The predicted molecular weights of acetamido-H’U₉H, H’U₉, and U₉ are 30,800, 25,400, and 24,100, respectively.](http://www.jbc.org/)

![Fig. 5. Effect of zinc ion on hydroxylamine-induced N-terminal cleavage of a mutant RecA intein. MU₁₉₉H was isolated as described under “Experimental Procedures” (lane 1) and incubated at 25 °C for 3 h in the presence of 1 mM TCEP and 0.5 M hydroxylamine, pH 7.0, and in the absence (lane 2) or presence (lane 3) of 2 mM ZnCl₂. The samples were then analyzed by SDS-PAGE together with molecular mass standards.](http://www.jbc.org/)
not possible to compare the splicing efficacy of the two intein variants. For the direct comparison of their splicing efficiencies, we synthesized both forms of the Synechocystis DnaE C-intein segment fused to the five N-terminal residues of the DnaE C-extein followed by a His tag (S\textsubscript{HC}\textsuperscript{H-35} and S\textsubscript{HC}\textsuperscript{H-H35}). Using an N-intein segment fused to MBP as the trans-splicing partner (MS\textsubscript{NA}), the extent of protein splicing after 16 h at pH 7.0 and 25 °C was 48% with S\textsubscript{HC}\textsuperscript{H-35} and 46% with S\textsubscript{HC}\textsuperscript{H-H35} (Fig. 6), indicating that the penultimate C-intein residue does not play a crucial role in protein splicing involving the DnaE intein.

**Effect of Zn\textsuperscript{2+} on Protein Splicing in trans by Wild-type and Mutant DnaE Intein Segments**—Protein splicing in trans by the DnaE split intein was also strongly inhibited by ZnCl\textsubscript{2}, with 80% inhibition at 0.2 mM ZnCl\textsubscript{2} for both S\textsubscript{HC}\textsuperscript{H-35} and S\textsubscript{HC}\textsuperscript{H-H35} (Fig. 7). Zn\textsuperscript{2+} was the most effective of the divergent transition metals ions tested followed by Cd\textsuperscript{2+}, whereas Co\textsuperscript{2+} and Ni\textsuperscript{2+} were less inhibitory, and Mg\textsuperscript{2+} had no significant effect (Table I).

The reversibility of the effect of Zn\textsuperscript{2+} on trans protein splicing by the DnaE split intein was examined by incubating the intein segments with 2 mM ZnCl\textsubscript{2} for 16.5 h at 25 °C. Under these conditions, protein splicing with the S\textsubscript{HC}\textsuperscript{H-35} and S\textsubscript{HC}\textsuperscript{H-H35} C-inteins in this experiment was inhibited 98 and 100%, respectively. Continued incubation at 25 °C for 20 h after the addition of 10 mM EDTA led to a significant level of protein splicing (83 and 94% of the amount expected in the absence of ZnCl\textsubscript{2} for S\textsubscript{HC}\textsuperscript{H-35} and S\textsubscript{HC}\textsuperscript{H-H35}, respectively), indicating almost complete reversal of Zn\textsuperscript{2+} inhibition.

**DISCUSSION**

Studies of the action of inhibitors on enzyme-catalyzed reactions have made important contributions to our understanding of enzyme mechanisms. This paper describes the first example of a general, reversible inhibitor of protein splicing. Relatively low concentrations of Zn\textsuperscript{2+} inhibited protein splicing mediated in trans by reconstituted fragments of the *M. tuberculosis* RecA intein (Fig. 3) and in cis by a RecA mini-intein (Fig. 4). Low concentrations of Zn\textsuperscript{2+} also inhibited hydroxylamine-induced N-terminal cleavage mediated in cis by a native RecA mutant mini-intein (Fig. 5) and in trans by reconstituted fragments of the RecA intein (Fig. 3). In all cases, the entire homing endonuclease domain had been deleted, eliminating it as a possible target for Zn\textsuperscript{2+} inhibition. Inhibition of protein splicing mediated in trans by a RecA intein reconstituted with a synthetic peptide lacking a His tag as the C-terminal fragment shows that not more than the 38 C-terminal intein residues are required for Zn\textsuperscript{2+} inhibition and that a His tag is not required. Inhibition of protein splicing by Zn\textsuperscript{2+} in a similar concentration range was also observed with the naturally split Synechocystis sp. PCC6803 DnaE intein (Fig. 7, Table I). The inhibition by Zn\textsuperscript{2+} of protein splicing mediated by two unrelated and quite different inteins suggests that Zn\textsuperscript{2+} may well be a general inhibitor of protein splicing.

The two trans-splicing systems employed for the *in vitro* study of Zn\textsuperscript{2+} inhibition were to some extent complementary. The RecA system is a more efficient protein-splicing system and is more versatile in that protein splicing depends less on the presence of adjacent natural extein amino acids than does the DnaE intein (6). However, its use is complicated by the need to reconstitute the intein by prior denaturation and renaturation. In this investigation, the RecA intein served as a useful system for comparing the inhibition of protein splicing with the inhibition of its first step, N-terminal cleavage, and for studying the reversibility of Zn\textsuperscript{2+} inhibition. On the other hand, the DnaE split intein system can be reconstituted without prior denaturation. This attribute greatly facilitated the systematic study of protein-splicing inhibitors such as the response to varying Zn\textsuperscript{2+} concentrations and the study of metal ion specificity. In addition, the DnaE system provides some insights into the role of the canonical His residue at the penul-
timate position at the intein C terminus, which has an Ala residue in its place (8). Our results indicated that the extent of protein splicing and the susceptibility to Zn$^{2+}$ inhibition are similar with Ala or His as the penultimate residue of the DnaE C-intein (Figs. 6 and 7).

The inhibition by Zn$^{2+}$ occurred at relatively low concentrations. Both the cis- and trans-splicing systems using the RecA intein were essentially completely inhibited by 2 mM ZnCl$_2$ (Figs. 3 and 4), and the trans-splicing system was essentially completely inhibited by 200 μM ZnCl$_2$ as well (cis splicing was not studied at lower Zn$^{2+}$ concentrations). The range of Zn$^{2+}$ concentrations that affected protein splicing in vitro is similar to the concentration range in which Zn$^{2+}$ modulates the activity of some E. coli enzymes, such as succinate oxidase (15), aminolevulinate dehydratase (16), and the YyrR phosphatase (17). It should be noted that all studies reported in this paper were carried out with TCEP rather than DL-1,4-dithiothreitol as the thiol reductant, thereby avoiding complications caused by the binding of Zn$^{2+}$ to the added thiol.

The specificity of metal ion inhibition was studied in the DnaE trans-splicing system. Zn$^{2+}$ was a significantly more potent inhibitor than other divalent transition metal ions (Table I). The presence of 2 mM ZnCl$_2$ inhibited protein splicing by almost 90%. Cadmium ion was only slightly less effective an inhibitor as Zn$^{2+}$, which is not surprising considering that cadmium has been shown to substitute functionally for zinc in other enzymes, such as carboxypeptidase (18) and β-lactamase (19). The only nontrans protein metal studied, Mg$^{2+}$, had no significant effect on protein splicing. The absence of an effect of Mg$^{2+}$ and the fact that protein splicing occurs effectively in vitro with intein components routinely refolded using buffers supplemented with 1 mM EDTA (4, 7) suggest that divalent metal ions do not play an essential role as cofactors in the protein-splicing reaction or as structural components of inteins. It is therefore unlikely that the inhibition by Zn$^{2+}$ involves competition with a natural divalent metal ion cofactor or prosthetic group.

The RecA intein was used to study the mode of zinc inhibition. Splicing was essentially completely inhibited by 2 mM ZnCl$_2$, and this inhibition was fully reversible upon the addition of 10 mM EDTA for 2.5 h at 25°C (Fig. 3). If inhibition by Zn$^{2+}$ involved the replacement of a tightly bound, essential metal ion, the addition of EDTA in the absence of the essential metal would not be able to effect its reversal. The first step of protein splicing, which can be assayed independently of subsequent steps by measuring the hydroxylamine-dependent N-terminal cleavage reaction (20, 21), was also completely inhibited by 2 mM ZnCl$_2$ in an EDTA-reversible manner in the trans-splicing system (Fig. 3). The initial step in protein splicing can also be studied in a cis-splicing system by using a mutant intein in which the Asn and Cys residues flanking the C-terminal splice junction, which are essential for steps 3 and 2 of protein splicing (for a recent review, see Ref. 1), have been replaced by Ala residues. Using such a mutant intein, the hydroxylamine-dependent reaction was more than 90% inhibited by 2 mM ZnCl$_2$ (Fig. 5). These observations indicate that Zn$^{2+}$ is a reversible inhibitor of protein splicing as well as of the initial N-S acyl rearrangement, the first step in protein splicing.

Since protein splicing in trans also requires prior reconstitution of the intein segments, one could argue that Zn$^{2+}$ interferes with the reassociation process rather than with protein splicing per se. At least two lines of evidence support the notion that Zn$^{2+}$ inhibits protein splicing directly. (i) Protein splicing in trans can be measured independently from reassociation by carrying out reconstitution in the absence of a thiol reductant, which leads to the formation of a disulfide-linked dimer that can undergo splicing in the presence of TCEP (4). The observation that subsequent TCEP-dependent protein splicing was inhibited by Zn$^{2+}$ implies that the metal ion does not remain present during intein refolding and assembly to exert its inhibitory effect. (ii) Unspliced precursor proteins involving the RecA intein, in which the second and third steps of the protein-splicing process, i.e., transelerification and Asn cyclization (see Fig. 4 of Ref. 1), are blocked owing to mutation of the amino acid residues flanking the C-terminal splice junction, can be isolated in the native state and used to study the first step of protein splicing in terms of hydroxylamine-dependent cleavage at the N-terminal splice junction (21). The observation that hydroxylamine-dependent cleavage of a fully folded mutant intein is inhibited by Zn$^{2+}$ (Fig. 5) demonstrates that the metal ion directly interferes with the first step of protein splicing.

A recent crystallographic analysis of the VMA intein of S. cerevisiae revealed a zinc ion chelated by the Cys at the C-terminal splice junction, the His residue adjacent to the intein C terminus, the Glu at position 80 in domain B, and a solvent water molecule (9). The relationship of this Zn$^{2+}$-complex to the inhibition of protein splicing by Zn$^{2+}$ described in this paper is not clear. It is worth noting that the effect of Zn$^{2+}$ on protein splicing mediated by the DnaE intein segments was not significantly affected by the absence or presence of His adjacent to the intein C terminus (Fig. 7). Also, the inhibition by Zn$^{2+}$ of the first step of protein splicing occurred in a RecA intein in which the Asn and Cys residues at the C-terminal splice junction were replaced by Ala. These observations suggest that the modes of Zn$^{2+}$ binding that exert inhibitory effects of Zn$^{2+}$ on the DnaE and the RecA inteins may not be the same as the mode of binding described by Poland et. al. (9).

The results presented in this paper constitute the first description of an inhibitor of protein splicing. The inhibition of protein splicing by Zn$^{2+}$ may be a relatively general phenomenon, as it is observed with two quite different inteins regardless of whether protein splicing occurred in cis or trans. The complete reversibility of this potent inhibition should make it a valuable tool in biochemical and biophysical studies of the mechanism of protein splicing. For example, it may allow the crystallization of protein-splicing precursors without the need for mutations to prevent splicing. The modulation of protein splicing by metal ions at concentrations only in slight molar excess over the intein concentration suggests the possibility that metal ions also could affect protein splicing in vivo and perhaps might even serve as a physiological mechanism to regulate protein splicing in certain organisms.

Acknowledgments—We thank Paul Morgan and Belinda Lew for helpful comments on the manuscript and Gina Pagani for synthesizing the peptides.

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J. Biol. Chem. 2001, 276:10832-10838.
doi: 10.1074/jbc.M011149200 originally published online January 10, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M011149200

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