Communication

Reengineering the Nucleotide Cofactor Specificity of the RecA Protein by Mutation of Aspartic Acid 100*

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We have recently obtained evidence for a direct link- age between the S_{0.5} (S_{0.5} is the substrate concentration required for half-maximal velocity) value of a nucleo side triphosphate and the conformational state of the RecA-ssDNA complex, with an S_{0.5} value of 125 \mu M or less required for stabilization of the strand exchange-active conformation. For example, although ATP and ITP are hydrolyzed by the RecA protein with the same turnover number (18 min^{-1}), ATP (S_{0.5} = 45 \mu M) functions as a cofactor for the strand exchange reaction, whereas ITP (S_{0.5} = 500 \mu M) is inactive as a strand exchange cofactor. The RecA protein crystal structure suggests that cofactor specificity is determined by Asp^{100}, which likely forms a hydrogen bond with the exocyclic 6-amino group of ATP; the higher S_{0.5} value for ITP is presumably due to unfavorable interactions between Asp^{100} and the 6-carbonyl group of the inosine ring. To test this hypothesis, we prepared a mutant RecA protein in which Asp^{100} was replaced by an asparagine residue. The S_{0.5} (ITP) for the [D100N]RecA protein is 125 \mu M, indicating favorable interactions between the Asp^{100} side chain and the 6-carbonyl group of ITP. Correspondingly, ITP functions as a cofactor for the strand exchange activity of the [D100N]RecA protein. This result demonstrates the importance of the residue at position 100 in determining nucleotide cofactor specificity and underscores the importance of the S_{0.5} value in the RecA protein-promoted strand exchange reaction.

The RecA protein of Escherichia coli (M, 37,842, 352 amino acids) is essential for homologous genetic recombination and for the postreplicative repair of damaged DNA. The purified RecA protein will promote a variety of DNA pairing reactions that presumably reflect in vivo recombination functions. The most extensively investigated DNA pairing activity is the ATP-dependent three-strand exchange reaction, in which a circular ssDNA molecule and a homologous linear dsDNA molecule are recombined to yield a nicked circular dsDNA molecule and a linear ssDNA molecule. This reaction proceeds in three phases. In the first phase, the circular ssDNA substrate is coated with RecA protein to form a presynaptic complex; this complex will catalyze the hydrolysis of ATP to ADP and P_i. In the second phase, the presynaptic complex interacts with a dsDNA molecule, the homologous sequences are brought into register, and pairing between the circular ssDNA and the complementary strand from the dsDNA is initiated. In the third phase, the complementary linear strand is completely transferred to the circular ssDNA by unidirectional branch migration to yield the nicked circular dsDNA and displaced linear ssDNA products (Roca and Cox, 1990; Kowalczykowski et al., 1994). The presynaptic complex formed between RecA protein and ssDNA is the active recombinational entity in the strand exchange reaction. The RecA protein binds cooperatively to ssDNA, forming a right-handed helical protein filament with one RecA monomer per four nucleotides of ssDNA and six RecA monomers per turn of the filament. In the absence of nucleotide cofactor or in the presence of ADP, the helical filament adopts a “collapsed” or “closed” conformation (helical pitch: 65 Å) that is inactive in strand exchange. In the presence of ATP or the nonhydrolyzable ATP analog, ATP\_γS, however, the filament assumes an “extended” or “open” conformation (helical pitch: 95 Å) that is active in strand exchange (Egelman, 1993).

We have been examining the mechanism of the nucleotide cofactor-mediated isomerization of the RecA-ssDNA complex and have identified a linkage between the S_{0.5} value\(^2\) of a nucleoside triphosphate and the conformational state of the RecA-ssDNA complex (Menge and Bryant, 1992; Meah and Bryant, 1993; Stole and Bryant, 1994, 1995). These studies have shown that a nucleoside triphosphate must have an S_{0.5} value of 100–120 \mu M or lower in order to stabilize the strand exchange-active conformation of the RecA-ssDNA complex. For example, although ATP and ITP are hydrolyzed by the RecA protein with identical turnover numbers (18 min^{-1}), ATP (S_{0.5} = 45 \mu M) functions as a cofactor for the strand exchange reaction, whereas ITP (S_{0.5} = 500 \mu M) is inactive as a strand exchange cofactor.

The x-ray crystal structure of the RecA protein-ADP complex indicates that cofactor specificity is determined by Asp^{100}, which forms a hydrogen bond with the exocyclic 6-amino group of adenosine base (Story et al., 1992); it seems likely that a similar contact is made with ATP in the RecA-ssDNA-ATP complex (Fig. 1), although no structural information is available for this complex. The higher S_{0.5} value for ITP, relative to that for ATP, is presumably due to unfavorable interactions between the negatively charged Asp^{100} side chain and the 6-carbonyl group of the inosine ring. To test this hypothesis, we prepared a mutant RecA protein in which Asp^{100} was replaced by an asparagine residue. The effect of this mutation on the nucleotide cofactor specificity of the RecA protein is described in this report.

EXPERIMENTAL PROCEDURES

Materials—Wild-type RecA protein was prepared as described previously (Cotterill et al., 1982). ATP and ITP were from Sigma, [\gamma^32P]ATP and [\gamma^32P]ITP were from ICN. [\gamma^32P]ITP was prepared

ATP\_γS, adenosine 5'-O-(thiotriphosphate).

1 S_{0.5} is the substrate concentration required for half-maximal velocity.

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The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; \delta X, bacteriophage \delta X174; SSB, E. coli single-stranded DNA-binding protein; PCR, polymerase chain reaction; ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate; ITP, inosine 5'-triphosphate.
Fig. 1. Active site interactions for the wild-type and [D100N]RecA proteins. Shown are proposed interactions between the 6-amino group of ATP and the side chain of aspartic acid 100 in the wild-type RecA protein (left) and between the 6-carbonyl group of ITP and the side chain of asparagine 100 in the [D100N]RecA protein (right).

Preparation of [D100N]RecA Protein—The mutant [D100N]RecA gene was produced by the polymerase chain reaction (PCR)-based overlap extension method essentially as described (Ho et al., 1989). The template for RecA mutagenesis consisted of a pUC19 vector containing a 1300-base pair E. coli DNA fragment carrying the wild-type recA gene and promoter cloned into a BamHI/HindIII site; the mutagenesis primers were 5'- CACCGCTGCAACCCACCTGC-3' and 5'-CGTAGATTGGTTCAGCCCGTAG-3' (the codon for Asn100 is underlined and the nucleotide mismatch is in bold). The resulting PCR fragment containing the mutant RecA gene and promoter region was cloned into pUC19 to yield the plasmid, pUCrecA[D100N]. The entire [D100N]RecA gene and promoter region was then introduced into the RecA deletion strain, BNN124, and the mutant [D100N]RecA protein was expressed and purified by methods that have been described previously (Bryant, 1988).

RESULTS AND DISCUSSION

Experimental Design—In an effort to reengineer the nucleotide cofactor specificity of the RecA protein, we prepared a new mutant RecA protein in which aspartic acid 100 was replaced by an asparagine residue. The expectation was that the D100N mutation would allow the protein to form hydrogen bonding interactions with the 6-carbonyl group of ITP that are not possible in the wild-type protein (Fig. 1). If the new interactions resulted in a significant decrease in the S_{0.5} value for ITP, we predicted that the mutation would convert the RecA protein into an ITP-activated DNA recombinase. The purified [D100N]RecA protein is shown in Fig. 2.

ssDNA-dependent NTP Hydrolysis Activity of the [D100N]RecA Protein—The ssDNA-dependent hydrolysis of ATP and ITP by the wild-type and [D100N]RecA protein was analyzed under standard reaction conditions (pH 7.5, 37°C). The dependence of the rate of ssDNA-dependent NTP hydrolysis on NTP concentration is shown in Fig. 3, and the steady-state kinetic parameters for the hydrolysis of each NTP are presented in Table I.

The turnover number (V_{max}/E_{0}) for ssDNA-dependent ATP hydrolysis by the [D100N]RecA protein was 18 min^{-1}, a value identical to that obtained for the wild-type RecA protein. The S_{0.5}(ATP) for the [D100N]RecA protein was 85 μM, approximately 2-fold higher than the value of 45 μM for the wild-type RecA protein. The ATP saturation curves were sigmoidal and identical Hill coefficients (n_H) of 3 were obtained for both proteins, indicating that both proteins are subject to positive cooperativity with respect to ATP concentration. These results show that the D100N mutation has a minimal effect on the steady-state kinetics of ssDNA-dependent ATP hydrolysis by the RecA protein.

The turnover number for ssDNA-dependent ITP hydrolysis by the [D100N]RecA protein was also 18 min^{-1}, a value again equivalent to that obtained for the wild-type protein. Moreover,
The ITP saturation curves were sigmoidal and identical Hill coefficients of 3 were obtained for both proteins, indicating that the ITP saturation curves were sigmoidal and identical Hill reaction. The small increase in the $S_{0.5}(\text{ITP})$ that results from the mutation indicates that the residue at position 100 contributes only to nucleotide cofactor binding in the strand exchange-active open conformation of the RecA-ssDNA complex.

Furthermore, our results show that the D100N mutation changes the $S_{0.5}$ value for nucleoside triphosphates, without affecting the turnover number for NTP hydrolysis or the cooperativity of binding of NTP to the RecA-ssDNA complex. This indicates that the residue at position 100 contributes only to cofactor recognition and not to the catalytic reaction and provides evidence to relate the $S_{0.5}$ value determined from the steady-state kinetics of ssDNA-dependent NTP hydrolysis with the binding affinity of the nucleotide cofactor for the NTP active site of the protein. The reduction of the $S_{0.5}(\text{ITP})$ from 500 to 125 $\mu$M upon mutation of Asp$^{100}$ to asparagine indicates that the 6-carbonyl group of ITP interacts more favorably with the Asp$^{100}$ side chain in the mutant protein than with the negatively charged Asp$^{100}$ side chain in the wild-type protein. The small increase in the $S_{0.5}(\text{ATP})$ that results from the mutation indicates that the 6-amino group of the adenosine ring can interact favorably with either an aspartic acid or asparagine at position 100, with the hydrogen bond between the negatively charged aspartic acid side chain being somewhat stronger than that with the neutral asparagine side chain.

The finding that the [D100N]RecA protein is able to use ITP as a cofactor for the three-strand exchange reaction also supports our proposal that it is not sufficient for an NTP cofactor to simply bind to and be hydrolyzed by the RecA protein, but that it also must bind with certain minimal affinity ($S_{0.5}$ of 125 $\mu$M or lower) in order to support isomerization of the RecA-ssDNA complex to the strand exchange-active open conformation.

**REFERENCES**

Bryant, F. R. (1988) J. Biol. Chem. 263, 8716–8723

Cotterill, S. M., Satterthwait, A. C., and Fersht, A. R. (1982) Biochemistry 21, 4332–4337

Cox, M. M., and Lehman, I. R. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 3433–3437

Egelman, E. H. (1993) Curr. Opin. Struct. Biol. 3, 189–197

Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Nature (Lond.) 343, 318–325

Kowalczykowski, S. C., Dixon, D. A., Eggleston, A. K., Lauder, S. D., Rehrauer, W. M. (1994) Microbiol. Rev. 58, 401–465

Menge, K. L., and Bryant, F. R. (1992) Biochemistry 31, 5151–5157

Roca, A. I., and Cox, M. M. (1990) Crit. Rev. Biochem. Mol. Biol. 25, 415–456

Stile, E., and Bryant, F. R. (1994) J. Biol. Chem. 269, 7919–7925

Stile, E., and Bryant, F. R. (1995) J. Biol. Chem. 270, 20322–20328

Storl, R. M., Weber, I. T., and Stelzl, T. A. (1992) Nature 355, 318–325

Weinstein, G. M., McEntee, K., and Lehman, I. R. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 126–130

**SUMMARY AND CONCLUSIONS**

Our results show that it is possible to alter the nucleoside triphosphate cofactor specificity of the RecA protein by mutating the aspartic acid residue at position 100 of the RecA polypeptide. This finding confirms that the hydrogen bonding interaction between Asp$^{100}$ and the 6-amino group of the adenosine ring that is apparent in the crystal structure of the RecA-ADP complex (which presumably represents the strand exchange-inactive closed conformation of the protein) also contributes to nucleotide cofactor binding in the strand exchange-active open conformation of the RecA-ssDNA complex.

The D100N mutation converts the RecA protein into an ITP-activated DNA recombinase.