Role of His-16 in Turnover of T4 Pyrimidine Dimer Glycosylase*

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Previously, the histidine residue at position 16 in the mature T4 pyrimidine dimer glycosylase (T4-PDG) protein has been suggested to be involved in general (non-target) DNA binding. This interpretation is likely correct, but, in and of itself, cannot account for the most dramatic phenotype of mutants at this position: their inability to restore ultraviolet light resistance to a DNA repair-deficient *Escherichia coli* strain. Accordingly, this residue has been mutated to serine, glutamic, aspartic acid, lysine, cysteine, and alanine. The mutant proteins were expressed, purified, and their abilities to carry out several functions of T4-PDG were assessed. The mutant proteins were able to perform most functions tested *in vitro*, albeit at reduced rates compared with the wild type protein. The most likely explanation for the biochemical phenotypes of the mutants is that the histidine residue is required for rapid turnover of the enzyme. This role is interpreted and discussed in the context of a reaction mechanism able to account for the complete spectrum of products generated by T4-PDG during a single turnover cycle.

The base excision repair (BER)$^3$ glycosylases initiate DNA repair by recognizing inappropriate or damaged DNA bases and removing them via glycosyl bond scission (1). These enzymes are responsible for the substrate specificity of their particular BER pathways, a formidable accomplishment, as this recognition must proceed within the context of a vast excess of perfectly normal and appropriate bases. A subset of these enzymes also carry out scission of the DNA sugar-phosphate backbone by β-elimination and adjacent to the site of glycosyl bond cleavage. These enzymes will be referred to as “BER glycosylase-lyases.” Enzymes in this subset of glycosylases utilize a primary or secondary amino acid group as an active site nucleophile. The amino group adds to C1' because of the developing electrophilic character of that carbon accompanying glycosyl bond cleavage. The resulting Schiff base enzyme-DNA intermediate, in its protonated form, has been proposed to kinetically assist DNA backbone cleavage (2). Gerlt (3) has hypothesized that the rate of the β-elimination reaction is directly determined by the acidity of the 2'-hydrogens on the sugar undergoing 3'-phosphate group elimination. Within this hypothesis, the role of the protonated Schiff base would be to lower the pKₐ of the 2'-hydrogens. In the proposed reaction scheme, one of these hydrogens would be abstracted by a general base, either an activated water or a basic amino acid side chain. T4-PDG is a ultraviolet light (UV) pyrimidine photodimer-specific BER glycosylase-lyase similar mechanistically to other BER glycosylase-lyases. The N-terminal threonine α-amino group is the active site nucleophile (4).

To investigate those amino acid candidates whose side chains might serve as general bases for abstracting a sugar 2'-hydrogen to initiate the β-elimination reaction, we identified all amino acid side chains within 10 Å of the α-amino nitrogen in the co-crystal structure of a T4-PDG mutant with its pyrimidine photodimer-containing substrate DNA (5). The most promising candidate was the His-16 residue. Augustine *et al.* (6) had mutated His-16 as part of a survey of the function of all the histidine residues within T4-PDG. The phenotypes of the various histidine mutants varied from little or no effect to the apparent severe loss of DNA repair capacity for His-16. The severe phenotype of His-16 mutants was of interest, although the drastic loss of function did not seem consistent with the proposed role as the general base abstracting a 2'-hydrogen: first, a water molecule should, in principle, be able to carry out the abstraction of the 2'-hydrogen in the absence of a side chain base, and second, the lyase (β-elimination) activity should be completely dispensable, as there are other cellular systems able to complement this function. These considerations indicated that, whereas His-16 seemed important, its role might be other than that hypothesized, e.g. it might be involved in enzyme turnover.

Because other work on this enzyme had indicated that T4-PDG was representative of the BER glycosylase-lyases in all steps subsequent to the glycosylase event, it was anticipated that the results obtained in this system might be readily extrapolated to other members of this enzyme class, e.g. the formamidopyrimidine DNA glycosylase and endonuclease VIII.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis at the His-16 Position—Mutagenesis at the His-16 position in the gene encoding T4-PDG that had been previously cloned into the pTYBII vector of the IMPACT system (interin-mediated purification with an affinity chitin-binding Tag, New England Biolabs) was performed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The success of the mutagenesis step was verified by DNA sequencing (Molecular Biology Core, NIEHS, University of Texas Medical Branch). The mutagenesis primers were obtained from the Midland Certified Reagent Company, Inc. (Midland, TX) and had the following sequences: 5'-GAATTGGCTGAACAAATYTTAATTG-GCTGAAATAT-3' and 5'-ATATTCGACCATTAAABCTTGGTGCAAGC-ATT3', and Table 1.

Primers were purified by gel electrophoresis through a 10% urea-formamide gel, eluted by suspension in buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA) for 1 h, purified using a BioSpin column (Bio-Rad), and dialyzed against TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) for 2 h.

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*∥* The abbreviations used are: BER, base excision repair; AP, abasic site.
pH 7.5, 1 mM EDTA) at 4 °C overnight. Concentrations of primer solutions were determined by absorbance measurements at 260 nm.

His-16 Mutant Protein Purification—pTBYHI plasmids containing the mutated sequences were transformed into Escherichia coli ER2566 cells (New England Biolabs). For each mutant, a 2-liter culture was grown at 37 °C until the absorbance at 600 nm reached 0.5, when induction was initiated with 0.3 mM isopropyl-β-D-galactopyranoside. The cultures were allowed to grow at 25 °C for 5 h and harvested by centrifugation at 5,000 × g for 10 min. The cell pellet was suspended in 200 ml of lysis buffer: 20 mM NaHEPES buffer, pH 7.0, 500 mM NaCl, 0.1 mM EDTA, 0.1% (v/v) Trition X-100, 0.8 μl/ml protease inhibitor (Sigma), and ruptured by a French pressure cell. Clarified extracts were prepared by centrifugation at 12,000 × g for 30 min. All mutants were stably expressed as evidenced by Western blot analysis with an antibody specific to T4-PDG (data not shown). A 10-ml column of chitin beads (New England Biolabs) was equilibrated with 10 column volumes of column buffer (20 mM NaHEPES buffer, pH 7.0, 500 mM NaCl, 0.1 mM EDTA), and the extract was loaded by gravity flow. The column was washed with 15 column volumes of column buffer, followed by a 5-column volume wash of high salt buffer (50 mM Tris-HCl, pH 8.0, 1 M NaCl, 1 mM EDTA). The column matrix was then equilibrated with 3 volumes of pre-cleavage buffer (20 mM NaHEPES buffer, pH 7.0, 50 mM NaCl, 0.1 mM EDTA), followed by a 3-volume wash with cleavage buffer (20 mM NaHEPES buffer, pH 7.0, 50 mM NaCl, 0.1 mM EDTA, 30 mM dithiothreitol), and allowed to equilibrate at 4 °C overnight. Protein was eluted with column buffer and collected in 3-ml fractions. Aliquots of column fractions were analyzed using an SDS-15% polyacrylamide gel. The purest fractions were combined, concentrated using Amicon YM10 filters (Millipore, Bedford, MA), and quantitated by Bradford assay (7). Concentrations ranged from 0.27 to 1.0 mg/ml. The proteins were dialyzed into 25 mM NaPO4 buffer, pH 7.0, 25 mM NaCl, 1 mM EDTA, and stored at 4 °C.

Measurement of UV-irradiated Cell Survival—Plasmids containing the genes encoding mutant and wild type enzymes were transformed into Escherichia coli AB2480 cells (wotA, recA), grown at 30 °C, and streaked onto Luria broth (LB) plates containing 100 μg/ml ampicillin. The plates were irradiated with a germicidal lamp (254 nm UV light) at 2.6 microwatts/cm², perpendicular to the axis of the streak. After growth at 30 °C overnight, the survival of the cells along the streak axis, hence along the gradient of UV light dosage, was evaluated.

Measurement of UV-irradiated Circular Plasmid DNA Nicking—pBR322 plasmid DNA solutions (0.3 μg/μl) in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA were irradiated with low pressure mercury vapor germicidal lamps (Millipore, Bedford, MA), and quantitated by Bradford assay (7). The number of observed single strand breaks introduced by each enzyme, not corrected for dilution, is displayed on the ordinate. The label adjacent to each curve designates the mutation at position 16, with + identifying the wild type enzyme. The concentration of pyrimidine dimers was 250 nM. The concentration of enzyme used in each reaction was (the letter indicates the amino acid mutation at position 16): + (0.52), A (0.35), D (0.04), C (0.02), E (0.014), K (0.012), and S (0.006).

The normalized initial velocities (strand breaks per DNA molecule per min per nx enzyme) were: + (0.52), A (0.35), D (0.04), C (0.03), E (0.014), K (0.012), and S (0.006).

Measurement of Abasic Site (AP) Oligodeoxynucleotide Nicking—An oligodeoxynucleotide with the sequence 5′-ACCATGCCTGCAAGAAU-TAACGAATTG-3′ and its complement, 5′-CATGGTAAATTTTTCTCGAG-GCATGTT-3′, were synthesized by the Midland Certified Reagent Co. The U-containing oligodeoxynucleotide was 32P-labeled at its 5′ end with T4 polynucleotide kinase (New England Biolabs) and annealed to its complementary strand. The resulting U-containing 26-mer duplex DNA Glycosylase-lyase Turnover

| Mutation | 5′-XYZ-3′ | 5′-ABC-3′ |
|----------|-----------|-----------|
| H16E     | AGC       | GCT       |
| H16D     | GAC       | GTC       |
| H16E     | GAA       | TTT       |
| H16K     | AAA       | TTT       |
| H16C     | TGT       | ACA       |
| H16A     | GCT       | AGC       |

FIG. 1. UV survival in cells expressing wild type and mutant T4-PDG enzymes. E. coli AB2480 cells, containing plasmids expressing wild type or mutant T4-PDG enzymes, or only the pBR322 vector, were streaked across LB plates and allowed to absorb onto the agar surface. The streaks were exposed to a gradient of 0–10 s of light from low pressure mercury vapor lamps. The incident fluence was 2.6 microwatts/cm² of short wavelength UV light. Ctrl indicates cells containing the vector plasmid alone, WT indicates cells expressing the wild type T4-PDG enzyme, and the streaks of cells expressing the various mutant genes are indicated as H16X, where the X indicates the amino acid (Ala, Cys, Asp, Glu, Lys, and Ser) substituted for histidine.

FIG. 2. Enzyme activity on UV-irradiated covalently closed circular plasmid DNA substrates. Solutions of plasmid DNA were irradiated with germicidal lamps as described under “Experimental Procedures.” The incident dose was adjusted to produce ~25 pyrimidine dimers per pBR322 DNA molecule. The enzyme reactions were carried out in a buffer containing 100 mM NaCl (distributive conditions; see the text). The number of observed single strand breaks introduced by each enzyme, not corrected for dilution, is displayed on the ordinate. The label adjacent to each curve designates the mutation at position 16, with + identifying the wild type enzyme. The concentration of pyrimidine dimers was 250 nM. The concentration of enzyme used in each reaction was (the letter indicates the amino acid mutation at position 16): + (0.42 nm), A (1.6 nm), C (12.5 nm), D (8.3 nm), E (31 nm), K (31 nm), and S (62 nm). The normalized initial velocities (strand breaks per DNA molecule per min per μg enzyme) were: + (0.52), A (0.35), D (0.04), C (0.03), E (0.014), K (0.012), and S (0.006).
DNA was incubated for 20 min at 37 °C with uracil DNA glycosylase (1 unit/pmol of DNA, New England Biolabs) in 25 mM NaHEPES buffer, pH 7.0, 10 mM NaCl, 100 μg/ml bovine serum albumin to form abasic sites (referred to as AP or AP sites). All substrates were prepared immediately before assays were carried out.

AP site-containing duplex oligodeoxynucleotide DNA (4 nM) was incubated for various times with several concentrations of wild type and mutant T4-PDG in a master mixture containing 25 mM NaHEPES buffer, pH 7.0, 10 mM NaCl, 100 μg/ml bovine serum albumin at 37 °C. Aliquots (10 μl) were quenched with 20 mM NaBH4 (freshly prepared on the day of use). An equal volume of loading buffer: 95% (v/v) formamide, 20 mM EDTA, 0.02% (w/v) bromphenol blue, 0.02% (w/v) xylene cyanol, 5% (w/v) SDS, was added to each aliquot, and the samples were heated to 90 °C for 5 min. The DNAs were loaded on a 15% polyacrylamide, 1% stacking gel in 1× TBE buffer (100 mM Tris, 100 mM boric acid, 2 mM EDTA, pH 8.9) containing 8 μl urea. The DNAs were separated by electrophoresis at 20 watts for 3 h. Gels were visualized by autoradiography using Kodak Scientific Imaging film and quantitated using PhosphorImager screens and ImageQuanti 5.0 software (Amersham Biosciences).

Measurement of Trapped Covalent Enzyme-DNA Complexes—AP site-containing duplex oligodeoxynucleotide DNA (4 nM) was incubated at 37 °C for 1 h with wild type or mutant T4-PDG enzymes in the presence of 100 mM NaCNBH3, for 1 h at 37 °C. The reactions were quenched with 10 mM NaBH4 for 5 min. The workup and analysis of all samples was as previously described.

AP-DNA (4 nM) was incubated for 5 min at 37 °C in a premixed solution of wild type or mutant enzyme (2–4 nM) and 100 mM NaCN. Reaction aliquots were quenched with 10 mM NaBH4 for 5 min at 37 °C. Parallel experiments were done in which 100 mM NaCN, 10 mM NaBH4, and enzyme were premixed, and then incubated with the DNA for 10 min at 37 °C. All reactions were worked up and analyzed as previously described.

RESULTS

Survival of DNA Repair-deficient E. coli Expressing Various His-16 Mutants—Fig. 1 shows the results of streak tests of several T4-PDG His-16 mutants. This test was useful to rapidly evaluate the biological consequence of enzyme mutants, as there was no capacity by these host cells to repair UV photodamage to DNA. Note that cells containing the plasmid expressing the wild type enzyme survived all doses of UV, whereas cells containing only the pBR322 vector plasmid, designated Ctrl, did not survive even moderate doses. The H16D-, H16C-, and H16A-expressing cells seemed to survive slightly better than the other mutants, but overall the relative survival of cells expressing any of the mutants was not greatly different from E. coli AB2480 cells containing only the vector plasmid.
Pyrimidine Dimer-specific Plasmid Incision—The plasmid nicking assay in Fig. 2 measured pyrimidine dimer glycosylase plus lyase activity. The results of this type of assay are sensitive to the salt concentration (8). At low salt, T4-PDG exhibits a “processive” nicking activity, in which a substantial fraction of the pyrimidine dimers in an individual DNA molecule were incised before the enzyme macroscopically dissociated from the vicinity of that DNA molecule. At high salt, the enzyme follows “distribution” kinetics, with a high probability of macroscopic dissociation from the included volume of the substrate DNA molecule following each incision event. When evaluating mutant enzymes for catalytic activity, distributive conditions (i.e., high salt) were found to give a kinetic behavior substantially devoid of the complications of processivity. Whereas the kinetics of the wild type enzyme continued to greater than 1.5 single strand breaks per molecule, all the mutants displayed qualitatively similar behavior to each other, in that they reached a plateau at an average of one or fewer incisions per DNA molecule. The amount of each enzyme used in this experiment was varied to obtain 0.3–1 single strand breakage events in 3–5 min. When normalized for the amount of enzyme used, the order of activity was found to be: \(+ > A \sim D > C \sim E \sim K \sim S\) (Fig. 2).

AP Site Oligodeoxynucleotide Incision—T4-PDG also incises DNA at an AP site by virtue of its lyase activity. This incision activity is illustrated in Fig. 3. Note that the reaction rates for all mutant enzymes slowed down, and in some cases, came to a plateau. The enzyme concentrations used in this series of experiments varied substantially; the concentration of H16A enzyme added was substantially lower than the other five mutants. The amount of wild type enzyme used was an additional 10-fold lower than the concentration of H16A. For all mutants the enzyme was in molar excess over the substrate, presumably reflecting single turnover conditions, but the proportion of active enzyme molecules was not measured for any of the proteins. Dr. Amanda McCullough\(^2\) has observed that the fraction of active T4-PDG molecules purified by the IMPACT method was only 20–40% of that of T4-PDG purified by a more traditional procedure (10). Clearly the H16A mutant retained more AP-lyase activity than the other mutants, but its behavior was qualitatively similar to them and distinct from that of the wild type enzyme. When normalized for the amount of enzyme used, the order of activity was found to be: \(+ > A > C \sim D > E \sim K \sim S\) (Fig. 3).

**Formation of Reducible Covalent Enzyme-Product Intermediates**—The AP-lyase activity of T4-PDG was shown to be mediated through a covalent enzyme-DNA imino (Schiff base) intermediate (4). This intermediate was identified by reduction with borohydride or cyanoborohydrde (11). Results shown in Fig. 4, panels A and B, demonstrated that all the mutants qualitatively retained the ability to form the imino intermediate, but their reaction rates were quantitatively altered from the wild type. Because of differences in their redox potential, the order of activity was found to be:

\[^{2}\text{A. McCullough, personal communication.}\]
borohydride effectively sampled a “snapshot” of the imino intermediates present at the time of addition (by reducing the aldehyde substrate as well as the imino intermediate), whereas cyanoborohydride, when present throughout the reaction, sampled the “accumulation” of intermediates (12), because it reduced the Schiff base, but not the aldehyde substrate within the duration of the experiment. Enzyme reactions mediated via an imino intermediate have been shown to be characteristically inhibited by cyanide, but only when the cyanide treatment was present during the reaction, i.e., pretreatment of the enzyme with cyanide, followed by removal of the reagent, resulted in no inhibition (4). The data in Fig. 4, panel C, demonstrated that the wild type enzyme and to a lesser degree the H16A mutant formed a covalent complex stabilized by cyanide. This difference from the other mutants was likely because of substantially reduced rates of Schiff base formation by the others. Cyanide inhibition likely measured a snapshot of the reaction, because cyanide would be expected to add to the aldehyde form of the AP substrate site to form a cyanohydrin.

Analysis of Enzyme Turnover—Because all the T4-PDG mutants at the His-16 position seemed able to carry out the glycosyl bond cleavage, Schiff base formation, β-elimination (and possibly δ-elimination) steps of the reaction, although at reduced rates, the biochemical basis of the observed DNA repair deficiency phenotype was unclear at this stage of the investigation. The one characteristic shared by all the mutants was a slowing of the reaction rate during the assay. This might have resulted from accumulation of some kind of inhibitory condition away from intermediates or products covalently bound to the enzyme. The quantitative product spectrum for a given reaction condition represents the balance among these rate constants (see the text).

b. **Suggested T4-PDG reaction mechanism.** The rate constant labels ($k_1$, $k_2$, $k_3$, $k_4$, and $k_5$) identify those forward rate constants leading to regeneration of product formation. At that time, either wild type or H16A mutant enzyme was added. The added H16A mutant enzyme was able to catalyze additional single strand break formation, but slowed in the second phase in a manner qualitatively similar to its behavior in the initial phase. As expected, wild type enzyme, added in the second phase in place of the H16A enzyme, was able to rapidly incise substrate DNA to completion.

**DISCUSSION**

One hypothesis tested in these experiments was whether His-16 was the general base responsible for initiating T4-PDG β-elimination by abstraction of a deoxyribose 2′ hydrogen. The rate of this proton abstraction is thought to determine the rate of the elimination reaction. The biochemical phenotypes of the mutant enzymes did not directly support this hypothesis. Instead, they were most consistent with His-16 being involved in turnover of one or more of the covalent enzyme-product species to regenerate the enzyme for another round of catalysis.

The cells containing plasmids expressing the various His-16 mutants were all highly susceptible to UV light inactivation, indicating that the mutant enzymes were not able to promote DNA repair nearly as efficiently as the wild type. This defect was not because of either a lack of expression or a complete loss of catalytic function, as all enzymes were capable of each of the component steps of catalysis: the glycosylase step, formation of the imino intermediate characteristic of this enzyme class, and the lyase step. The rates of these reactions were lower than the wild type for all the mutant enzymes (except H16A, see below), which may partially explain the biological phenotypes. In general the mutant enzymes displayed similar phenotypes whether the plasmid pyrimidine dimer assay or the oligonucleotide AP site assay was used. The sole exception was the H16A mutant. It had essentially wild type activity in the plasmid assay (activity measured as the initial velocity of the reaction), whereas its activity in the AP site assay was only 10% that of the wild type enzyme. A possible explanation for this discrepancy lies in the salt concentration difference between the two assays. The His-16 residue has been shown to affect the processivity of T4-PDG (6). The plasmid assay was carried out at high salt to minimize the effect of processivity, whereas the AP site assay was done in low salt. If the intrinsic...
glycosylase activity of H16A was not different from wild type, the salt difference (through its effect on the rate of enzyme-DNA complex formation) could explain this difference. In any case, even the H16A mutant, the most active of the mutants on a per molecule basis, was clearly incapable of turnover to regenerate a fully competent catalytic species after some number of β- and/or δ-elimination events. This lack of turnover was not because of accumulation of some inhibitory species, because the mutant and wild type enzymes were able to carry out further incision events on the product DNA generated by H16A. The wild type enzyme was able to carry out many additional catalytic rounds.

Why should the rates of all steps be reduced in the mutants compared with the wild type enzyme, when their primary defects appear localized to enzyme turnover? In the case of the H16D, H16E, and H16K mutants, this may be because of a perturbation of the electrostatics of the active site. However, if that is correct, the biochemical phenotypes of the H16A, H16C, and H16S enzymes could not be explained by the same mechanism, particularly because the H16A enzyme was the least defective of the mutant enzymes. Alanine should be devoid of side chain functional properties including large electrostatic effects. The phenotype of these three mutants may be partially because of the small size of their side chains. This may allow a water molecule(s) to take over some of the histidine functionality. The more drastic defects of H16C and H16S compared with H16A may be partially explained by their hydrogen bonding ability in that these side chains may inappropriately orient the water molecule(s) putatively substituting for the histidine functionality.

A suggested T4-PDG reaction mechanism is diagrammed in Fig. 6. In this scheme, the product spectrum (by which we mean the proportions of AP sites, β-elimination products, and δ-elimination products) of an initial catalytic encounter between the enzyme and its substrate would be a consequence of the probabilities of product formation within the reaction scheme. These probabilities would be, in turn, functions of the rate constants: $k_1$, $k_2$, $k_3$, $k_4$, and $k_5$. For this initial encounter reaction, the probability of formation of an AP site would be $k_1/(k_1 + k_2)$, the probability of formation of a β-elimination product would be $[k_2/k_1 + k_2][k_3/k_3 + k_4]$, and the probability of formation of a δ-elimination product would be $[k_2/k_1 + k_2][k_4/k_3 + k_4]$. The rate of enzyme turnover after a δ-elimination event would be proportional to the hydrolysis rate constant $k_5$.

Alternative schemes might be envisioned in which the formation of β-elimination and/or δ-elimination products might require a second (or even third) encounter with the enzyme subsequent to the initial glycosyl bond cleavage reaction. Unless active sites for those reactions are completely different from those implied in Fig. 6, any such alternative schemes must violate microscopic reversibility; all such mechanisms require an obligatory hydrolysis and dissociation of the Schiff base intermediate and/or the E-SSB species of Fig. 6 as a consequence of the multiple encounter requirement. Because microscopic reversibility ensures that the transition states for a reaction and its reverse are the same, reassociation of the enzyme with product DNA to form either the Schiff base intermediate or the E-SSB cannot just regenerate exactly the species as depicted in Fig. 6, and still be consistent with the assumed requirement for multiple encounters. The T4-PDG literature does not contain any suggestion of such multiple active sites.

The original hypothesis tested in this investigation proposed a role for His-16 in the step labeled by $k_5$ in Fig. 6. Whereas the rate of this step may be modulated by the residue at position 16, no evidence directly supporting the original hypothesis was found. Instead, a more likely interpretation is that His-16 is involved in one or more of the steps labeled by $k_1$, $k_3$, and $k_5$. The rates of the other reactions involving the Schiff base intermediate and the intermediate labeled E-SSB, common to the $k_1$, $k_3$, and $k_5$ reactions, may also be influenced by His-16 functions. To our knowledge, this is the first reported identification of a residue involved in lyase product turnover for a BER glycosylase-lyase enzyme.

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