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Specificities and Kinetics of Uracil Excision from Uracil-Containing DNA Oligomers by *Escherichia coli* Uracil DNA Glycosylase†

Umesh Varshney and J. H. van de Sande*
Department of Medical Biochemistry, The University of Calgary, Calgary, Alberta T2N 4N1, Canada
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ABSTRACT: Uracil DNA glycosylase excises uracil residues from DNA that can arise as a result of deamination of cytosine or incorporation of dUMP residues by DNA polymerase. We have carried out a detailed study to define the specificities and the kinetic parameters for its substrates by using a number of synthetic oligodeoxyribonucleotides of varying lengths and containing uracil residue(s) in various locations. The results show that the *Escherichia coli* enzyme can remove a 5′-terminal U from an oligomer only if the 5′-end is phosphorylated. The enzyme does not remove U residues from a 3′-terminal position, but U residues can be excised from oligonucleotides with either pd(UN)p or pd(UNN) 3′-termini. The oligomer d(UUUUT) can have the second or third U residues from the 5′-end excised even when the neighboring site is an abasic site (3′ or 5′, respectively). On the basis of these findings, pd(UN)p was anticipated to be the smallest size substrate. Results show detectable amounts of U release from the substrate pd(UT)p; however, significantly higher amounts of U release were observed from pd(UT-sugar) or pd(UTT). Determinations of the K_m and V_{max} values show that the different rates of U excision from oligomers of different sizes (trimeric to pentameric) but containing U in the same position are largely due to the differences in the K_m values, whereas the different rates of U excision from the substrates of the same size but containing U in different positions are largely due to different V_{max} values.

The DNA glycosylases excise damaged or unconventional bases from DNA and initiate the DNA base excision repair pathway (Friedberg et al., 1978). These enzymes have been identified and purified from both prokaryotic and eukaryotic sources (Friedberg et al., 1978; Duncan, 1981; Lindahl, 1982; Morgan & Chlebek, 1989). Uracil DNA glycosylase excises uracil residues from DNA that can arise as a result of either deamination of deoxycytosine or incorporation of dUMP

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*To whom correspondence should be addressed.

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residues by DNA polymerase. None of the uracil DNA glycosylases studied so far require metal ions for their activity, and both single-stranded and double-stranded DNA containing uracil are used as substrates. However, the enzyme from most sources has higher activity with single-stranded DNA than with double-stranded DNA. The uracil DNA glycosylases are product inhibited by free uracil and a limited number of its derivatives, e.g., 6-aminouracil, 5-azauracil and 5-fluorouracil. The uracil DNA glycosylases have been shown to excise some of the analogues that are effective as inhibitors if they are incorporated into DNA. Another category of inhibitors of uracil DNA glycosylases is represented by *Bacillus subtilis* phage PBS2 and *Escherichia coli* phage T5 induced proteins (Cone et al., 1980; Warner et al., 1980; Wang & Mosbaugh, 1988). The amino acid sequences of the uracil DNA glycosylases deduced from the DNA sequence of the *E. coli*, yeast, and the putative human mitochondrial *ung* genes show a high degree of sequence homology in several regions of the protein, suggesting a conservation of structure and function relationship of the protein (Varshney et al., 1988; Percival et al., 1989; Olsen et al., 1989). In order to further understand the mechanism of action of the enzyme, we report on the substrate specificities and the kinetics of the *E. coli* uracil DNA glycosylase for a number of oligodeoxyribonucleotides of different sizes and containing U in varying positions.

Materials and Methods

Oligodeoxyribonucleotides. Oligodeoxyribonucleotides were obtained from the Regional DNA Synthesis Laboratory, University of Calgary, Calgary, Canada, and were resuspended in water at a concentration of $0.1 \times n$ OD mL^{-1}, where n is number of deoxyribonucleotides in the oligomer (approximately 10 pmol μL^{-1}).

5'-End Labeling, Purification, and Quantitation of Oligomers. Approximately 500 pmol of each oligodeoxyribonucleotide was 5'-end phosphorylated at 37 °C for 30 min in a 60-μL reaction consisting of 50 mM glycine buffer (pH 9.5), 10 mM DTT, and 5 mM $MgCl_2$ and an excess of low specific activity [γ-^{32}P]ATP (2.5 μmol) and T4 polynucleotide kinase (7.5 units). Under these conditions quantitative end labeling was achieved (Chaconas & van de Sande, 1980). Low specific activity [γ-^{32}P]ATP was prepared by diluting 2.0 μL of 7000 Ci/mmol (22 μM) [γ-^{32}P]ATP (ICN) with 150 μL of 100 μM cold ATP solution (λ_{max} 259 nm, ϵ 15 400). Reactions were terminated by heating at 90 °C for 5 min. The end-labeled oligodeoxyribonucleotides were purified by use of NENSORB20 cartridges (New England Nuclear) as follows. The hydrophobic resin of the column was packed with methanol and equilibrated with buffer A [100 mM Tris-HCl (pH 7.7), 1 mM Na_2EDTA, and 10 mM triethylamine]. Reactions were diluted to 300 μL with buffer A and loaded onto the column. The column was washed once with 200 μL of buffer A and once with 200 μL of distilled water. The oligodeoxyribonucleotides were eluted with 400 μL of 50% methanol, lyophilized, and resuspended in 500 μL of H_2O.

The oligomers were purified free of protein, salt, unincorporated radioactive nucleotides, and other low molecular weight materials and used as substrates for uracil DNA glycosylase. Only in the case of the dimer pd(UT) was the recovery low (~10%); in all other cases it was better than 90% (data not shown).

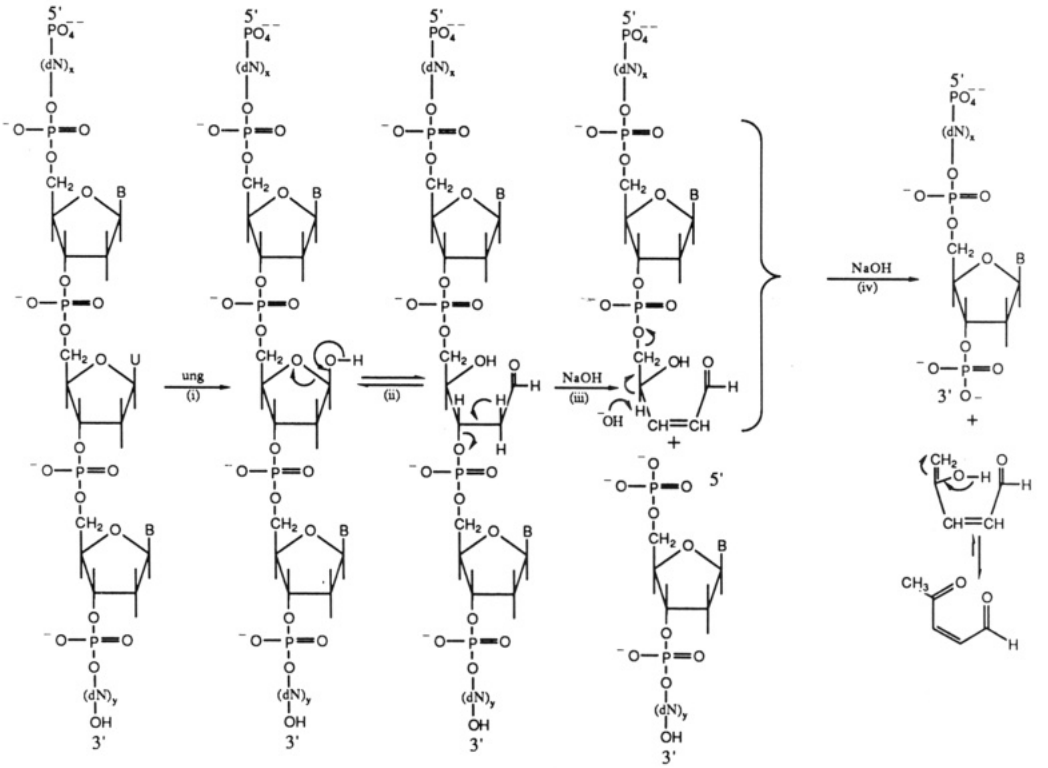
Uracil DNA Glycosylase Reactions. (a) *E. coli* uracil DNA glycosylase used in these studies was purified to homogeneity, and reactions were carried out under previously established conditions (Lindahl et al., 1977; Varshney et al., 1988) with minor modifications. In brief, 0.2 pmol of the ^{32}P 5' end-labeled oligodeoxyribonucleotides in 15 μL of reaction buffer [70 mM Hepes/KOH (pH 7.4), 1 mM Na_2EDTA, 1 mM DTT, and 25 μg/mL enzyme grade bovine serum albumin (Boehringer Mannheim)] were mixed with 5 ng of uracil DNA glycosylase and the mixture was incubated at 37 °C for 30 min. The reaction was stopped by adding 15 μL of 0.1 M NaOH and by chilling the reaction in wet ice. The phosphodiester bonds at the abasic sites were then cleaved by heating at 90 °C for 30 min. The contents of the tube were lyophilized and resuspended in 30 μL of the sample loading buffer (80% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol FF, and 1 mM Na_2EDTA). Control reactions for each oligodeoxyribonucleotide reaction were treated the same except that no enzyme was added. Aliquots (5.0 μL) were electrophoresed on 18% polyacrylamide 7 M urea gels (Maxam & Gilbert, 1980) of 0.75-mm thickness, and the gels were autoradiographed for 2 to 4 h at room temperature by use of intensifying screens and Kodak XAR films.

(b) Post-end-labeling uracil DNA glycosylase reactions were performed on 5'-unphosphorylated (–OH) oligodeoxyribonucleotides. Approximately 0.5 pmol of the oligomer was reacted with uracil DNA glycosylase as above and neutralized with 0.1 N HCl (15 μL) following the alkaline treatment at 90 °C. The reactions were divided into two parts, and one part was treated with 1 unit of calf intestinal alkaline phosphatase at 37 °C for 30 min. The phosphatase was denatured by three cycles of heating at 90 °C for 5 min and freezing in liquid N_2 for 2 min. Aliquots (5.0 μL) from both parts were taken and 5'-end labeled at 37 °C for 30 min in the presence of 20 μCi of [γ-^{32}P]ATP (~300 Ci/mmol) and 1 unit of T4 polynucleotide kinase in a 15-μL reaction buffer as described above. After the polynucleotide kinase was inactivated by heating at 90 °C for 5 min, the contents of the reaction were lyophilized and resuspended in 10 μL of sample loading buffer. Aliquots (2–3 μL) were analyzed on 18% polyacrylamide 7 M urea gels (Maxam & Gilbert, 1980).

Preparation of pd(UT-sugar) and pd(UT)p. Forty picomoles of 5' ^{32}P end-labeled pd(UTA) was suspended in 20 μL of 75% formic acid/5% piperidine and the solution incubated overnight at 37 °C. Piperidine formate was removed from the reaction by 3 cycles of lyophilization and resuspension in distilled water. Half of this reaction containing pd(UT-sugar) was taken up in 50 μL of 0.1 M NaOH and heated at 90 °C for 0.5 h to remove the sugar residue from the 3'-end to yield pd(UT)p. The pd(UT-sugar) and pd(UT)p were gel purified from an 18% polyacrylamide gel following autoradiography. Uracil DNA glycosylase reactions were performed as described above.

Determinations of K_m and V_{max} Values. Enzyme reactions using varying amounts of 5' ^{32}P end-labeled oligodeoxyribonucleotides (0.25–45 pmol) were taken up in 10 μL of reaction buffer and supplemented with 5.0 μL of the appropriate enzyme dilution prepared in the reaction buffer. The reactions were incubated for 20 min at 37 °C and analyzed on polyacrylamide gels along with a lane of 0.1 pmol of the low specific activity ATP. Gel pieces corresponding to the substrate, product, and ATP bands were cut out and counted by liquid scintillation counting. The amounts (in picomoles) of the product and substrate bands were calculated by use of the counts of ATP as a standard. Velocity (v) was presented as picomoles of product released per minute per nanogram of protein. Substrate concentration [S] was calculated as nanomoles per liter. The K_m and V_{max} values were determined from Hofstee plots (Dowd & Rigg, 1965) of at least two independent determinations.

Scheme I



RESULTS

Characterization of the Oligodeoxyribonucleotides. Approximately 0.05 pmol of the oligodeoxyribonculeotides d(UT), d(UTT), d(UTTT), d(UTTTT), d(TUT), d(TUTT), d(TTUT), d(TTTUG), d(UUUUT), and d(T_5UT_5) was 5′ ^{32}P end phosphorylated in the presence of excess [γ-^{32}P]ATP, analyzed on 18% polyacrylamide 7 M urea gels, and autoradiographed (Figure 1). Bands corresponding to [γ-^{32}P]ATP and [^{32}P]P_i are also marked. The migration of the oligodeoxyribonucleotides of such short lengths on polyacrylamide gels is base-composition dependent. Under these conditions, U migrates slightly faster than T and therefore the pentamer pd(UUUUT) migrates slightly faster than the pentamer pd(UTTTT). All other oligomers containing single U residues migrate according to their sizes.

Substrate Specificities. Uracil DNA glycosylase catalyzes glycosidic bond scission at U residues, leaving abasic sites that are sensitive to cleavage by heating at 90 °C under alkaline conditions. In general, two steps of β-elimination reactions take place (Scheme I). During the first step (reaction iii), hydrolysis of the phosphodiester bond at the abasic site results in two products: a 5′-side product possessing a sugar moiety (semialdehyde) at the 3′-terminus and a 3′-side product possessing a 5′-phosphate. During the second step (reaction iv) of the β-elimination reaction, the sugar residue from the 3′-end of the 5′-side product is removed, leaving a 3′-phosphate. If the abasic site was generated right at the 5′-end of the oligomer, the first step of the β-elimination reaction as above will lead to two products, sugar-P_i (from the 5′-side) and pd(n − 1) (from the 3′-side). A second round of β-elimination, which generates free P_i from sugar-P_i, proceeds at a slow rate due to an additional negative charge at the 5′-PO_4, and under the experimental conditions this elimination does not go to completion. Thus, three reaction products are obtained, two of which (originating from the 5′-side) will be radioactive.

The 5′ ^{32}P end-labeled oligodeoxyribonucleotides were tested as substrates for the *E. coli* uracil DNA glycosylase. Figure 2 shows the results of this experiment. Reaction of uracil DNA glycosylase with pd(UTT), pd(UTTT), and pd(UTTTT) results in bands corresponding to the expected products, sugar-P_i and P_i. Since none of these bands were present in the control reactions, this shows that the uracil residues from these oligomers were excised by the enzyme. The uracil residue from pd(UTT) was only partially excised, leaving a significant amount of the unreacted oligomer (Figure 2). Thus, pd(UTT) served only as a poor substrate. An even shorter oligomer, pd(UT), showed no detectable levels of products (Figure 2).

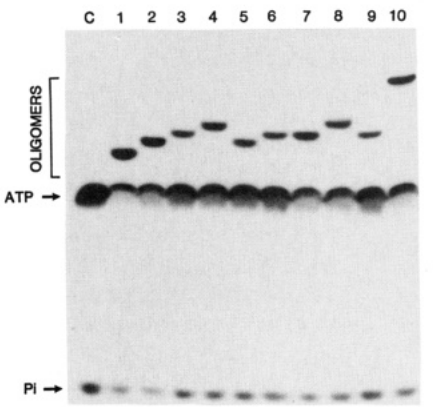


FIGURE 1: 5′-End ^{32}P phosphorylation of oligodeoxyribonucleotides. Oligodeoxyribonucleotides were 5′-end labeled with use of T4 polynucleotide kinase and low specific activity [γ-^{32}P]ATP, analyzed on a urea polyacrylamide gel, and autoradiographed. The lanes represent (1) pd(UT), (2) pd(UTT), (3) pd(UTTT), (4)pd(UTTTT), (5) pd(TUT), (6) pd(TUTT), (7) pd(TTUT), (8) pd(TTTUG), (9) pd(UUUUT), and (10) pd(T_5UT_5).

The oligonucleotides pd(TUTT) and pd(T_5UT_5) were also substrates for the enzyme, resulting in the formation of labeled pdTp and pd(T_5)p. The faster mobility of these products relative to the 5′-end-phosphorylated oligomers of the same sizes is due to the presence of an additional phosphate group at the 3′-terminus. The oligomer pd(UUUUT) upon reaction with uracil DNA glycosylase produced four new bands, P_i,

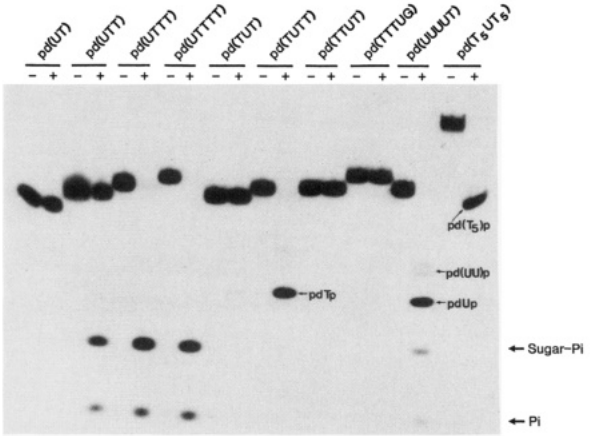


FIGURE 2: Determination of substrate specificity. The 5′ ^{32}P end-labeled oligodeoxyribonucleotides were incubated with (+) or without (−) *E. coli* uracil DNA glycosylase. Products (following alkaline treatment) were analyzed on a urea polyacrylamide gel and autoradiographed.

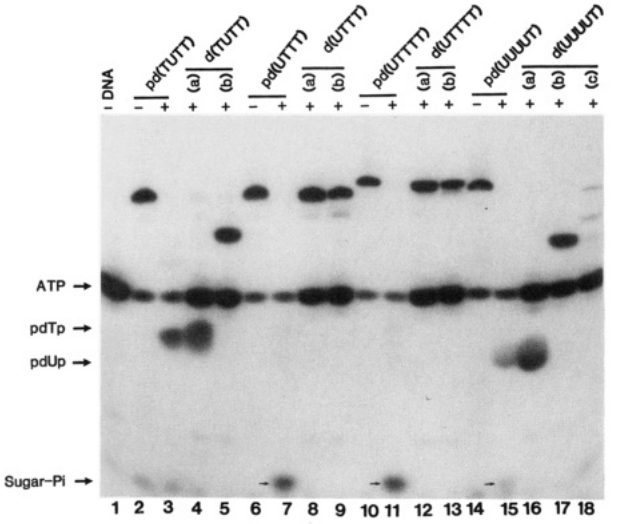


FIGURE 3: Effect of 5′-end phosphate on substrate specificity. Reactions were performed and analyzed as in Figure 2 except that in lanes marked +(a), +(b), or +(c) unlabeled oligomers (i.e., 5′-OH) were first reacted with uracil DNA glycosylase and the products analyzed by 5′-end labeling with T4 polynucleotide kinase and [γ-^{32}P]ATP as follows: (a) end labeling was performed without any phosphatase treatment, (b) 5′-end labeling was performed following treatment with calf intestinal alkaline phosphatase, (c) the same procedure was used as in (b) except 1/100 dilution of the uracil DNA glycosylase was used.

sugar-P_i, pdUp, and pd(UU)p, corresponding to the cleavage of U residues at the first, second, and third positions (from the 5′-end). No band corresponding to the excision of U at the fourth position was seen, suggesting that the enzyme is unable to remove U located at the penultimate position from the 3′-OH end. Three other oligomers, pd(TUT), pd(TTUT), and pd(TTTUG), possessing U at this position also did not serve as substrates for the uracil DNA glycosylase (Figure 2). It is also interesting to note that the intensity of the band corresponding to the cleavage of the U residue at position 2 (from the 5′-end) in pd(UUUUT) is much higher than the intensities of the bands corresponding to the cleavage of U residues in the first and third positions. This observation suggests that the rate of cleavage of the second U position is higher than the rate of cleavage at the first and third positions. Alternatively, this may also be due to the ability of the enzymes to cleave U in the second position even if the third position is an abasic site. Since the products were identified from the 5′-labeled end, the intensity of the band corresponding to cleavage at the second position may, therefore, appear high. This was further investigated by performing 5′ ^{32}P end labeling of the uracil DNA glycosylase reaction products from unphosphorylated oligomers following their treatment with calf intestinal alkaline phosphatase. Products corresponding to the 5′-side as well as to the 3′-side of the abasic site can be identified by this procedure, provided that they can be 5′ ^{32}P end labeled by T4 polynucleotide kinase (minimum size substrate requirement is Np) as shown below.

Effect of a 5′-End PO_4 Group on Excision of 5′-Terminal U Residues. Both 5′-[^{32}P]PO_4 and 5′-[OH] oligomers were reacted with uracil DNA glycosylase and heated under alkaline conditions. Products of 5′-OH oligomers were divided into two parts. One part was 5′ ^{32}P phosphorylated directly, whereas the other part was 5′ ^{32}P phosphorylated following treatment with calf intestinal alkaline phosphatase. Phosphatase treatment prior to end labeling assisted in the 5′ ^{32}P end labeling of the products that arose from the 3′-side of the abasic site. Excess [γ-^{32}P]ATP was not separated from the reactions prior to their analysis on polyacrylamide gel. Bands corresponding to free [γ-^{32}P]ATP and other radioactive contaminants were identified by running an aliquot of radioactive ATP on the gel (lane 1, Figure 3). Bands corresponding to P_i are not shown, as there was a significant amount of [^{32}P]P_i in the ATP making them indistinguishable from this background. Lanes 2 and 3, 6 and 7, 10 and 11, and 14 and 15 (Figure 3) are essentially repeats from Figure 2, and they were included as controls to provide markers to assist in the identification of various reaction products. The oligomers d(UTTT) (lanes 8 and 9) and d(UTTTT) (lanes 12 and 13) revealed only one band corresponding to the 5′ ^{32}P end-labeled unreacted substrates (compare lanes 6 and 10), indicating that the U residue from the 5′-terminal positions of the 5′-unphosphorylated oligomers could not be excised. Excision of the U residue from the second position was, however, still observed in these oligomers. This is demonstrated by the reaction of the uracil DNA glycosylase on d(TUTT) by the presence of pdTp when the products were 5′-end labeled (lane 4) and by the presence of pd(TT) when the products were treated with alkaline phosphatase prior to their 5′-end labeling (lane 5). The absence of a band corresponding to dTp is because the alkaline phosphatase treatment changes it to dT, which is no longer a substrate for T4 polynucleotide kinase. Similarly, observation of a pdUp band in lane 16 (Figure 3) suggests that the second U residue from d(UUUUT) was also excised by the enzyme. A band for pd(UU)p corresponding to U excision at the third position was unlikely to be visible because this band is usually very faint (see Figure 2). Furthermore, the 3′-phosphatase activity of the T4 polynucleotide kinase, which has a minimum size requirement of NNp (Cameron & Uhlenbeck, 1977), may also have converted a fraction of pd(UU)p to pd(UU), making detection difficult. When excess enzyme (5 ng) was used, a single band corresponding to U excision at the third U (from the 5′-end) was observed (lane 17, Figure 3), which is represented by the pd(UT) product. Absence of other bands in this lane suggests that U in the second position must also have been completely excised. However, at a lower enzyme concentration (50 pg), a band corresponding to cleavage at the second U (from the 5′ end) was also seen in addition to a third band corresponding to the unreacted substrate (lane 18, Figure 3). Excision at the third position may be expected to give rise to two products represented by a band originating from the 5′-sequences of the abasic site, pd(UU), and a band originating from the

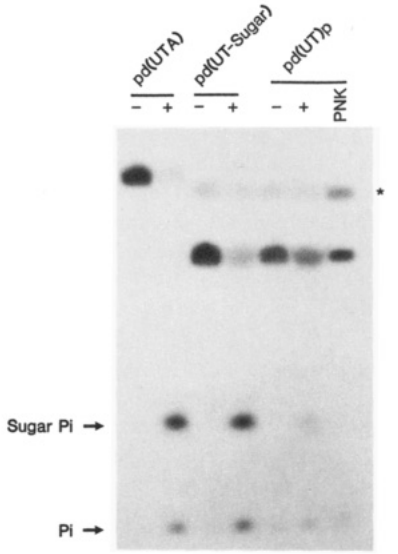


FIGURE 4: Effect of 3′-end phosphate and minimum size requirement for *E. coli* uracil DNA glycosylase. Reactions (+) and (−) were the same as in Figure 2. The reaction marked PNK shows results of p(UT)p treatment with T4 polynucleotide kinase.

3′-sequences of the abasic site, pd(UT). The presence of the band pd(UT) is clearly seen. In longer exposures, the presence of a band corresponding to pd(UU) and migrating slightly ahead of pd(UT) was also seen (data not shown). In addition, intensity of the product (trimer) corresponding to cleavage at the second U (from the 5′-end) was more than the intensity of the band corresponding to cleavage at the third U (from the 5′-end), suggesting a higher rate of excision of U at the second position. It is therefore conceivable that most U residues in the second position (from the 5′-end) of this oligomer will be excised prior to the completion of the excision of the U residues in the third position (from the 5′-end). Yet, at a higher enzyme concentration (lane 17) the product corresponding to complete excision of the U residues from the third position was observed. These observations suggest that excision of U residues can take place even if the neighboring position is an abasic site. In these reactions (lanes 17 and 18, Figure 3), again no product corresponding to cleavage at the first U position (from the 5′-end) i.e., tetramer pd(UUUT), was seen.

Minimum Substrate Size Requirement and Effect of the 3′-PO_4 Group on Substrate Specificity. Results presented in Figures 2 and 3 show that U located as the third nucleotide from the 3′-OH end could be excised by the enzyme, but not when located second from the 3′-OH end. Results presented elsewhere (Delort et al., 1985) showed that U located at the 3′-terminal position was not a substrate. Therefore, a 5′-phosphorylated trimer possessing U at the 5′-terminal position, i.e., pd(UNN), should be a substrate. As is shown in Figure 2, pd(UTT) was cleaved by uracil DNA glycosylase, although at a low efficiency. It was also shown that pd(UT) was not a substrate (Figure 2). To further investigate the minimum substrate size requirement of *E. coli* uracil DNA glycosylase, a new oligomer pd(UTA) was synthesized, 5′-end labeled, purified, and converted to pd(UT-sugar) and pd(UT)p. As expected, pd(UTA) is a substrate and two new bands corresponding to sugar-P_i and P_i are seen as a result of the enzyme activity; pd(UT-sugar) and pd(UT)p also show the sugar-P_i and P_i bands as a result of enzyme action, suggesting that they are also substrates (Figure 4). However, the intensity of these bands was lowered significantly when the substrate was shortened to pd(UT)p, suggesting it to be a poorer substrate (Figure 4). In this experiment, the band marked with an asterisk was also seen when pd(UT-sugar) and pd(UT)p were used as substrates. This band was not due to nonspecific activity of the uracil DNA glycosylase because it was also present in the control reactions where no enzyme was used. Since pd(UT-sugar) and pd(UT)p were purified as single bands from a gel, the mechanism of its origin is unclear. The intensity of this band increased when pd(UT)p was treated with T4 polynucleotide kinase, suggesting that the band corresponded to pd(UT) because the 3′-phosphatase activity of the T4 polynucleotide kinase will lead to conversion of pd(UT)p to pd(UT). These results suggest that an oligomer with U at the second position from the 3′-end must have a phosphate at the 3′-end to allow *E. coli* uracil DNA glycosylase activity at this position.

Table I: K_m and V_{max} Values for Uracil-Containing DNA Oligomers

oligomer	K_m[a] ($\times 10^{-7}$ M)	V_{max}[b]
pd(UTT)	14.80 ± 0.57	0.185 ± 0.01
pd(UTTT)	1.74 ± 0.08	0.25 ± 0.04
pd(UTTTT)	1.32 ± 0.14	0.28 ± 0.03
pd(TUTT)	2.20 ± 0.14	1.3 ± 0.03
pd(T_5UT_5)	1.29 ± 0.16	18.8 ± 1.8

[a] K_m values are for the uracil residue in the oligomer. [b] V_{max} values are in picomoles per minute per nanogram of protein.

Kinetics of U Excision. Kinetic determinations (K_m and V_{max} values) on five substrates were performed to further understand the mechanism of U excision by the *E. coli* uracil DNA glycosylase. Range finding experiments suggested that the enzyme concentrations of 50, 5, 5, 2, and 0.25 ng mL^{-1} were suitable to study the kinetics of pd(UTT), pd(UTTT), pd(UTTTT), pd(TUTT), and pd(T_5UT_5), respectively. These experiments also suggested the following order to efficiency for U residue cleavages: pd(T_5UT_5) >> pd(TUTT) > pd(UTTTT) > pd(UTTT) >>> pd(UTT) (data not shown). The products of the reactions from six to eight different substrate concentrations ranging from 0.2 to 4 μM for pd(UTT), 0.05 to 2 μM for pd(UTTT), 0.05 to 1.5 μM for pd(UTTTT), 0.5 to 2 μM for pd(TUTT), and 0.025 to 1 μM for pd(T_5UT_5) were run on polyacrylamide gels. Gel pieces corresponding to the product and undigested substrate bands were cut out and counted. A band corresponding to 0.1 pmol of ATP was also cut out and counted and used as an internal standard for the quantitation of products and substrate concentrations. The velocity (v) was calculated as picomoles of product released per minute per nanograms of protein. The substrate concentration [S] was calculated by the addition of the quantities of the product and the unused substrate and expressed in nanomoles per liter. The values of K_m and V_{max} were calculated from a Hofstee linear transformation of the Michaelis–Menten type kinetics by plotting values of v (ordinate) vs v/[S] (abscissa). Intercepts on the ordinates represent V_{max} values, whereas the slopes of the lines represent K_m. Average values of K_m and V_{max} of at least two independent determinations are shown in Table I.

Data presented in Table I suggest that the K_m values for the uracil residue in the oligomers are size dependent. It drops dramatically when the oligomer size is increased from a trimer (K_m 14.8 $\times 10^{-7}$ M) to a tetramer (K_m (1.74–2.20) $\times 10^{-7}$ M). Further increase in size to a pentamer or undecamer does not cause such dramatic changes in K_m values. The differences in the efficiency of excision of the U residues from a trimer, a tetramer, and a pentamer possessing U in the same position (from the 5′-terminus) are largely due to differences in K_m values, as their V_{max} values are quite similar. Thus, a very high K_m value for pd(UTT) compared to those of pd(UTTT) and pd(UTTTT) may explain why it is a poor substrate. On the

other hand, two tetrameric substrates, pd(UTTT) and pd(TUTT), with similar K_m values but with significantly different V_{max} values suggest that the location of the U residue is critical in making the pd(TUTT) a better substrate than pd(UTTT). Oligomer pd(T_5UT_5) was the optimal substrate studied because of its high V_{max} (18.8 pmol min^{-1} ng^{-1} of protein). These results suggest that the substrate size-dependent differences in the efficiency of U-residue excision are largely due to K_m values, whereas the differences in the efficiency of U excision, which are position dependent, are largely due to V_{max} values.

Discussion

Substrate specificities of *E. coli* uracil DNA glycosylase for a number of synthetic oligodeoxyribonucleotides (dimer to undecamer) containing U in different positions show that the uracil residue could be excised by the *E. coli* enzyme from the following substrates: pd(UTT), pd(UTTT), pd(UTTTT), pd(TUTT), and pd(T_5UT_5). Uracil residues in the first, second, and third positions (from the 5'-end) in the 5'-phosphorylated pentameric substrate pd(UUUUT) could be cleaved. However, the uracil residues from pd(UT), pd(TUT), pd(TTUT), and pd(TTTUG) could not be excised by the *E. coli* enzyme. Failure of excision of the U residue from pd(UT) could be due to both the position of U and the size of the oligonucleotide. However, the failure to excise the U residue in the other oligonucleotides must be due to its location in these oligomers. The uracil residue in all of these oligodeoxyribonucleotides is located at the second position from the 3'-OH end. In fact, the second U residue from the 3'-OH end of the pentanucleotide pd(UUUUT) could not be excised. Therefore, the *E. coli* enzyme is unable to excise U residues when located second from the 3'-OH end of the oligomers. An earlier study (Delort et al., 1985) using octadeoxyribonucleotides also suggested that when a U residue was located at the 3'-terminus or at the second position from the 3'-OH end, it was not excised by the *E. coli* or the *Micrococcus luteus* enzymes. Our experiments suggest that the second U residue from a 3'-end could, however, be excised by the *E. coli* enzyme if the oligonucleotide possessed a phosphate group at the 3'-terminus.

We have also studied the effect of the 5'-PO_4 group on the substrate specificities. End labeling of the oligomers d(UTT), d(UTTT), d(UTTTT), and d(UUUUT) after treatment with uracil DNA glycosylase clearly suggested that the 5'-end must be phosphorylated for the 5'-terminal U residue to be excised. Excision of U residues in the second or other locations was not affected by the absence of the 5'-PO_4 group. These studies, therefore, show the unphosphorylated tetramer containing a U residue at the second position (from the 5'-end) to be a minimum substrate. This may explain why in previous studies the tetrameric substrate d(UUUU) was reported to be the shortest substrate for the *E. coli* uracil DNA glycosylase (Duncan, 1981; Lindahl, 1982).

In addition, our results show a trimer to be the minimum substrate if the oligomer is phosphorylated at the 5'-end and a dimer when both ends are phosphorylated, provided the U residue is located at the 5'-terminal position. Minimal cleavage of U by the *E. coli* uracil DNA glycosylase from trimers obtained from pancreatic DNase I degraded PBS1 DNA (i.e., containing a phosphate group at the 5'-end and a hydroxyl at the 3'-end) was also reported previously (Lindahl et al., 1977). Our results suggest that the uracil residues in these oligomers must have been located at the 5'-terminal position. The report that U residues located in the 5'-terminal position of a 5'-phosphorylated octamer are excised by the *M. luteus* but not the *E. coli* enzyme (Delort et al., 1985) is in contradiction with our findings. Differences in the kinetic parameters of the excision of U located in different positions of their mixed oligomers could account for this discrepancy. Also, a comparison of d(UUUUT) and pd(UUUUT) indicated that uracil at substrate positions can be excised even though the neighboring site is an abasic residue.

The K_m value for uracil excision in the trimeric substrate pd(UTT) was found to be 14.8×10^{-7} M, but it decreases in the tetrameric to undecameric oligomeric substrates (2.2×10^{-7} M to 1.29×10^{-7} M). These K_m values, as may be expected, are quite different from the *E. coli* enzyme K_m values for uracil residues in natural substrates such as phage PBS2 DNA ($K_m = 4 \times 10^{-8}$ M). As seen in Table I, K_m values contribute largely to the rate of U excision from similar locations on different size oligomers, whereas the V_{max} values are largely responsible for the position-dependent differences.

An earlier study (Leblanc et al., 1982) suggested that the *M. luteus* enzyme does not act in a processive mode. Results using pd(UUUUT) as a substrate show that bands corresponding to cleavage of U residues in the first, second, and third positions could be obtained independently of each other (Figures 2 and 3). While these observations may suggest a random mode of U excision by the *E. coli* enzyme, they do not rule out the possibility of the enzyme acting in a processive fashion because the substrate is not sufficiently long. Our studies on the effects of PO_4 groups at the 5'- and 3'-ends of the oligomers, however, do lead to further refinement of the definition of minimum size requirement of the substrate pd(UT)p for the *E. coli* enzyme. It must be noted, however, that it is a very poor substrate when compared to pd(UTA) or pd(UT-sugar). The efficiency of uracil excision from the latter two did not appear to be significantly different, which suggests that it is the length of the sugar–phosphate backbone that is more critical for the enzyme in at least the primary recognition of its substrate.

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Registry No. pd(UTT), 132540-81-9; pd(UTTT), 132540-77-3; pd(UTTTT), 132540-78-4; pd(TUTT), 132540-79-5; pd(T_5UT_5), 132540-80-8; uracil DNA glycosylase, 59088-21-0.

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Cryoenzymic Studies on Actomyosin ATPase: Kinetic Evidence for Communication between the Actin and ATP Sites on Myosin†

C. Tesi,‡ K. Kitagishi,§ F. Travers, and T. Barman*
INSERM U128, CNRS, BP 5051, 34033 Montpellier Cedex, France
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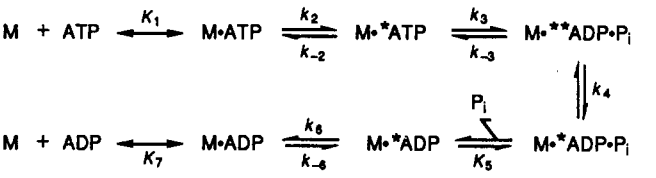
ABSTRACT: The post-ATP binding steps of myosin subfragment 1 (S1) and actomyosin subfragment 1 (actoS1) ATPases were studied at −15 °C with 40% ethylene glycol as antifreeze. The cleavage and release of P_i steps were studied by the rapid-flow quench method and the interaction of actin with S1 plus ATP by light scattering in a stopped-flow apparatus. At −15 °C, the interaction of actin with S1 remains tight, and the K_m for the activation of S1 ATPase is very small (0.3 μM). The chemical data were interpreted by E + ATP → E*·ATP ↔ E**·ADP·P_i → E*·ADP → products, where E is S1 or actoS1. In P_i burst experiments with S1, there was a large P_i burst of free P_i, but E**·ADP·P_i could not be detected. Here the predominant complex in the seconds time range is E*·ATP and in the steady-state E*·ADP. With actoS1, there was a small P_i burst of E**·ADP·P_i, evidence that the cleavage steps for S1 and actoS1 are different. From the stopped-flow experiments, the dissociation of actoS1 by ATP was complete, even at actin concentrations 60× its K_m. Further, no interaction of actin with the key intermediate M*·ATP could be detected. Therefore, at −15 °C, actoS1 ATPase occurs by a dissociative pathway; in particular, the cleavage step appears to occur in the absence of actin. It is proposed that the actin-induced conformation of myosin (M^+) is very sensitive to the temperature: under "normal" conditions, it isomerizes rapidly to the M* conformation which would explain the near-identity of the cleavage step of S1 and actoS1, but at −15 °C, this isomerization is slow, so the chemical step occurs with the M^+ conformation which could explain the difference of the chemical steps. A reaction scheme for actoS1 is proposed which is based on this and already published data.

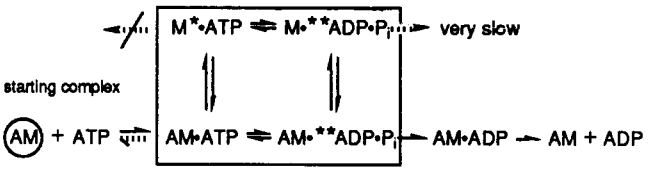
Muscle contraction depends on the cyclic interaction of actin and myosin, the energy for which is supplied by the hydrolysis of adenosine 5′-triphosphate (ATP)[1] by the myosin heads. It is thought that movement is controlled by actin interacting in different ways with the different intermediates on the myosin ATPase reaction pathway.

The kinetic processes of organized systems are difficult to study directly. Most work on actomyosin has been confined to solution studies using purified myosin—or more often its proteolytic fragments subfragment 1 (S1) or heavy meromyosin (HMM)—and actin [e.g., see Taylor (1979), Hibberd and Trentham (1986), and Cooke (1986)]. Further, experimental protocols have been dictated more by the physicochemical properties of the individual proteins, such as their solubilities and interactions, than by the conditions prevailing in vivo. Certainly a detailed knowledge of the physicochemical properties of the several proteins making up muscle is indispensable toward a full understanding of muscle contraction.

Scheme I



Scheme II



However, any extrapolation of this knowledge to the interpretation of phenomenon observed with, e.g., muscle fibers must be done selectively and with caution.

It is generally agreed that for myosin alone in solution the ATPase pathway is described by the Bagshaw–Trentham

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‡ Permanent address: Dipartimento di Scienze Fisiologiche, Università degli Studi di Firenze, I-50134 Firenze, Italy.
§ Permanent address: Otsuka Electronics Co., Ltd., 3-26-3 Shodai-Tajika, Hirakata, Osaka 573, Japan.

[1] Abbreviations: ATP, adenosine 5′-triphosphate; P_i, inorganic orthophosphate; S1, myosin subfragment 1; HMM, heavy meromyosin; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol.

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