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Kinetic Mechanism of Human Apurinic/Apyrimidinic Endonuclease Action in Nucleotide Incision Repair

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Abstract—Human major apurinic/apyrimidinic endonuclease (APE1) is a multifunctional enzyme that plays a central role in DNA repair through the base excision repair (BER) pathway. Besides BER, APE1 is involved in an alternative nucleotide incision repair (NIR) pathway that bypasses glycosylases. We have analyzed the conformational dynamics and the kinetic mechanism of APE1 action in the NIR pathway. For this purpose we recorded changes in the intensity of fluorescence of 2-aminopurine located in two different positions in a substrate containing dihydrouridine (DHU) during the interaction of the substrate with the enzyme. The enzyme was found to change its conformation within the complex with substrate and also within the complex with the reaction product, and the release of the enzyme from the complex with the product seemed to be the limiting stage of the enzymatic process. The rate constants of the catalytic cleavage of DHU-containing substrates by APE1 were comparable with the appropriate rate constants for substrates containing apurinic/apyrimidinic site or tetrahydrofuran residue, which suggests that NIR is a biologically important process.

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Key words: APE1, nucleotide incision repair (NIR), kinetic mechanism

Ionizing and ultraviolet radiation and various chemical mutagens constantly act on the cells of living organisms. During metabolic processes in cells, reactive oxygen species are generated. This affects first of all the cellular DNA, causing damage that leads to cell death, tumors, autoimmune diseases, and aging. To protect the genetic information, the cells of living organisms possess repair systems that correct DNA damage. Damaged heterocyclic bases in DNA are mainly eliminated through base excision repair (BER) pathway [1]. This repair pathway is started by activities of DNA glycosylases that eliminate damaged or mismatched bases through producing apurinic/apyrimidinic sites (AP sites). AP sites also appear as a result of spontaneous loss of bases (mainly purines) [1-3]. AP sites can inhibit DNA replication and transcription. In the case of replication, the newly synthesized DNA chain mainly incorporates adenosine opposite the AP site [4], and this results in single-nucleotide substitution in DNA. During BER, DNA is cleaved from the 5′-terminus of the AP site by apurinic/apyrimidinic endonucleases (AP endonucleases). In humans, APE1 (35.5 kDa) is the major AP endonuclease [5, 6]. It was recently shown that repair of some types of oxidative base damage can occur with involvement only of AP endonucleases and without glycosylases [7-12]. This process was termed nucleotide incision repair (NIR) [7]. During NIR, AP endonuclease catalyzes hydrolysis of the phosphodiester bond on the 5′-terminus of the damaged deoxynucleotide. The break in DNA chain results in production of a hydroxyl group on the 3′-end of the break and of phosphate group on the 5′-end. The production of a 3′-hydroxyl group promotes the further repair synthesis of DNA. The damaged dangling nucleotide can be removed by flap endonuclease 1 [13]. During NIR no AP sites, which are potentially toxic intermediates, are produced [7]. In human cells the NIR pathway involves AP endonuclease APE1 [9]. APE1 was shown to cleave DNA containing 5,6-dihydropyrimidines, α-2′-deoxyadenosine, α-thymidine, α-2′-deoxy-

Abbreviations: APE1, human apurinic/apyrimidinic endonuclease; AP site, apurinic/apyrimidinic site; 2-aPu, 2-aminopurine; BER, base excision repair; DHU, 5,6-dihydrouridine; F, tetrahydrofuran; NIR, nucleotide incision repair; Trp, tryptophan.

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cytidine, 5-hydroxy-2′-deoxyuridine, and 5-hydroxy-2′-deoxycytidine [9, 11, 12].

Using a stopped flow instrument and recording changes in the fluorescence of tryptophan residues within APE1 [14], we have studied the conformational dynamics of the full-length APE1 and of the truncated NΔ61-APE1 lacking first 61 N-terminal amino acids (the REF1 domain) in the BER and NIR pathways during their interactions with a DNA substrate containing a 5,6-dihydrouridine (DHU) residue. 5,6-Dihydropyrimidines are mainly produced in DNA under the influence of γ-radiation in the absence of oxygen [15]. The REF1 domain is necessary for NIR to provide for efficient formation of a complex with the substrate.

To comprehend in more detail the mechanism of APE1 action in the NIR pathway, in the present work conformational changes in DNA substrates were studied. We introduced into DHU-containing substrates a fluorescent analog of the heterocyclic base 2-aminopurine (2-aPu). Within DNA the fluorescence of 2-aPu residues was quenched due to aromatic stacking of 2-aminopurine with the neighboring bases in DNA. Formation of hydrogen bonds and solvation with water did not influence the 2-aPu fluorescence [16]. The stacking of 2-aPu with aromatic residues of the protein was also associated with quenching of its fluorescence [17]. Disorders in the aromatic stacking of 2-aminopurine increased the fluorescence intensity [18, 19].

New data from the present work have shown that the rate of hydrolytic cleavage of a DHU-containing DNA substrate by APE1 during nucleotide incision repair is comparable to the rate of cleavage of a DNA substrate containing an apurinic/apyrimidinic site during base excision repair. Thus, nucleotide incision repair together with base excision repair significantly contributes to maintaining genome stability.

MATERIALS AND METHODS

Enzyme and oligonucleotides. APE1 was isolated from *E. coli* cells and purified as described in [9]. Deoxyribooligonucleotides d(ATGCACATCGTCTACATGCGTATGCAGTCA), d(TGACTGCAT**XYZ**CATGTAGACGATGTGCAT) (X = 2-aPu, Y = DHU or C, Z = G; or X = A, Y = DHU, Z = G or 2-aPu) were synthesized in the Laboratory of Bionanotechnology (Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences) by the standard phosphoramidite method and purified by anion-exchange and reversed-phase HPLC. Concentrations of single-stranded deoxyribooligonucleotides were determined spectrophotometrically, and their extinction coefficients were calculated using the "nearest–neighbor" method [20]. The 5′-terminus of oligonucleotides d(TGACTGCATXYGCATGTAGACGATGTGCAT) (X = A or 2-aPu, Y = DHU) was labeled with ^{32}P according to a standard method [21] using T4-polynucleotide kinase (10-20 U; SibEnzyme, Russia) and [γ-^{32}P]ATP (Biosan, Russia). The following structures of double-stranded oligonucleotides were used:

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5′ TGACTGCATA(DHU)GCATGTAGACGATGTGCAT 3′   DHU/G
3′ ACTGACGTAT—G—CGTACATCTGCTACACGTA 5′

5′ TGACTGCAT(2-aPu)CGCATGTAGACGATGTGCAT 3′   (2-aPu)C/G
3′ ACTGACGTA——T——GCGTACATCTGCTACACGTA 5′

5′ TGACTGCAT(2-aPu)(DHU)GCATGTAGACGATGTGCAT 3′   (2-aPu)DHU/G
3′ ACTGACGTA——T———G—CGTACATCTGCTACACGTA 5′

5′ TGACTGCATA(DHU)(2-aPu)CATGTAGACGATGTGCAT 3′   DHU(2-aPu)/G
3′ ACTGACGTAT—G———C——GTACATCTGCTACACGTA 5′
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Substrate cleavage experiments. Time dependences of the cleavage of specific substrates DHU/G and (2-aPu)DHU/G labeled at the 5′-terminus were obtained in the presence of 1 μM APE1 and at substrate concentrations of 0.5 or 0.75, 1, and 1.25 μM. The reaction was performed at 25°C in BER buffer (20 mM HEPES/KOH, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$) or in NIR buffer (20 mM HEPES/KOH, pH 6.8, 50 mM KCl, 0.01 mM $MgCl_2$) for the DHU-containing substrate; in NIR buffer for the substrate containing 2-aPu and DHU. The enzyme was rapidly added to the corresponding ^{32}P-labeled substrate. The reaction was terminated at different time intervals by addition of reaction mixture aliquots into a solution containing 7 M urea, 0.1% bromophenol blue, and 0.1% xylene cyanol FF. The cleavage products were separated in 20% polyacrylamide gels. The gels were dried, visualized using a Molecular Imager FX phosphorimager (Bio-Rad, USA), and processed using the GelPro Analyzer software (Media Cybernetics, USA). The data were analyzed with the DynaFit program (BioKin, USA) [22].

Fluorescence titration. The emission fluorescence of 2-aPu-containing substrates was recorded at emission wavelength λ_{em} = 370 nm with excitation wavelength λ_{ex} = 310 nm. The maxima of fluorescence excitation and emission of the tryptophan residues of APE1 were, respectively, λ_{ex} = 281 nm and λ_{em} = 330 nm [14].

Under steady-state conditions, the APE1 (25 μM) was titrated with undamaged ligand (2-aPu)C/G (2.5-100 μM), and the 2-aPu fluorescence was measured to determine the fraction of active APE1. For the control, the dependence of the 2-aPu fluorescence on the ligand concentration was previously obtained in the absence of APE1. The measurements were performed in an 80-μl thermostatic cuvette at 25°C using a Cary Eclipse spectrofluorimeter (Varian, Australia) in the NIR buffer. The glycerol concentration in the solution during measurements was ~11%.

To assess the affinity of APE1 for the substrate DHU/G, the enzyme was titrated with the substrate under steady-state conditions. Each point on the fluorescent titration curve was obtained by measurement of the

APE1 fluorescence intensity in separate solutions (80 μl) containing 1 μM enzyme and the substrate (0.5-20 μM). Mixtures of the enzyme and substrate were incubated separately for 10 min at 25°C in the NIR buffer. Then the enzyme was mixed with the substrate, and the fluorescence of the enzyme was recorded with the Cary Eclipse spectrofluorimeter. The time from the mixing to recording of the fluorescence was about 30 sec. Within this time ~15% of the substrate was cleaved, thus most of the substrate (~85%) was unchanged. The dissociation constant (K_d) was calculated based on the dependence of the enzyme fluorescence intensity on the substrate concentration as described in [14].

Stopped-flow experiments. The pre-steady-state kinetics of the interaction of APE1 with the DNA substrates was studied using the stopped-flow method. The kinetic curves were recorded at 25°C with an SX.18MV stopped-flow spectrometer (Applied Photophysics, Great Britain) detecting the fluorescence signal of either protein tryptophan residues or 2-aPu substrate residues. The fluorescence of tryptophan residues was measured at wavelengths >320 nm using a WG320 light filter. The fluorescence of 2-aPu was detected at wavelengths >370 nm using an LG-370 light filter. The dead time of the device was 1.38 msec; in all calculations data obtained at times ≥2 msec were used. The reaction was performed under single-turnover conditions in the BER and NIR buffers. On recording the fluorescence of the tryptophan residues of the protein, the APE1 concentration in the reaction cell was 1 μM, and the concentration of the oligonucleotide substrate was varied from 0.75 to 2.0 μM. On recording the 2-aPu fluorescence the substrate concentration in the reaction cell was 1 μM, and concentration of APE1 was varied from 0.75 to 1.5 μM. Every kinetic curve for short times is a result of averaging of four or more experimental curves. For very long times no averaging was performed. The data were processed using the nonlinear regression analysis with the DynaFit program [22]. The kinetic curves were analyzed as described earlier [14].

RESULTS AND DISCUSSION

APE1 cleavage of ^{32}P-labeled DHU-substrate. Kinetic curves of product accumulation in APE1-catalyzed cleavage of DHU/G substrate ^{32}P-labeled at the 5′-terminus were obtained in the NIR and BER buffers (Fig. 1). During 35 h, the enzyme cleaved >60% of the DHU-containing substrate in both buffers. The same result was obtained for 2-aPu-containing substrate ((2-aPu)DHU/G) (figure not shown) in the NIR buffer. This indicates that the 2-aPu residue does not influence the cleavage of DHU-containing substrate by APE1, and therefore under our conditions it can be used as a fluorescent reporter group.

On kinetic curves of product accumulation (Fig. 1) three phases could be observed. Within the first phase (<20 sec) the cleavage by APE1 of the DHU-containing substrate (Fig. 1) shows a "burst" step of product accumulation in both the BER and NIR buffers. During the next two phases the products accumulate more slowly. Note that in both kinetic series (Fig. 1) the concentration of products generated during the first phase of the process ("burst") was lower than the initial concentration of the enzyme. In both cases (Fig. 1) on the "burst" phase accu-

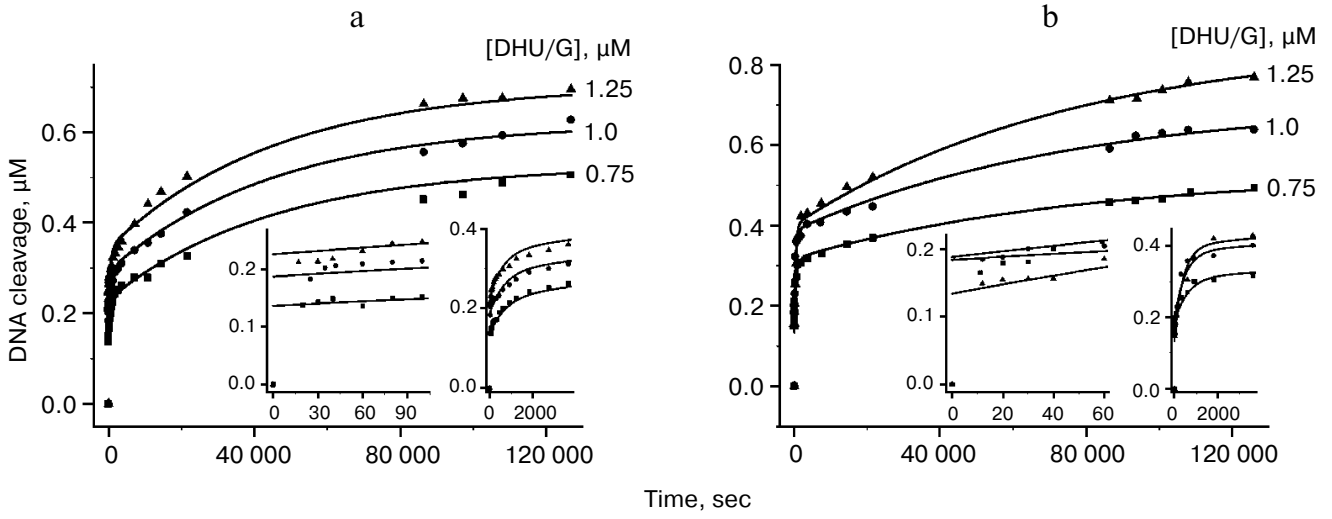


Fig. 1. Cleavage of DHU/G substrate by APE1 (1 μM) in BER (a) and NIR (b) buffers. The substrate concentrations are presented on the right axis. On the left inserts initial regions of the kinetic curves are shown that correspond to termination of phase 1, a "burst" in the accumulation of cleavage products within the time interval <20 sec. In the right insets kinetic curves are shown which correspond to phase 2 observed at $20 < t < 2000$ sec. Phase 3 corresponds to the slow accumulation of cleaved DNA at $t > 2000$ sec.

mulation of product was ~15% of the initial concentration of the DHU-substrate. The second phase of accumulation of the product was observed at $20 < t < 2000$ sec. The third phase corresponded to an extremely slow accumulation of the product and continued for >2000 sec. The slow biphasic accumulation of the product suggests that the reaction could occur with a less active form of the enzyme and that a stable complex of the enzyme with the product could exist.

Assay of APE1 activity. The lower amplitude of the "burst" in accumulation of cleavage products at the stage of catalysis (Fig. 1) compared to the total enzyme concentration could be, in particular, explained by the absence of completely active enzyme. Using the gel-retardation method, we revealed that no less than 64% of APE1 molecules (1 μM) bound with the DHU-containing DNA ligand (5 μM) (data not shown). This method underestimates the amount of bound DNA [23]. To exactly determine the active enzyme fraction, we titrated APE1 with an undamaged ligand (2-aPu)C/G containing 2-aPu. We concurrently recorded changes in the fluorescence intensity of 2-aPu on the binding of the ligand with the enzyme. The increase in intensity of 2-aPu fluorescence due to increase in the concentration of the ligand with 2-aPu was prevented by correcting the base line (see "Materials and Methods"). The APE1 concentration was much higher than the dissociation constant of the enzyme complex with the ligand (2-aPu)C/G and was 25 μM (K_d = 2.07 μM in the NIR buffer, data not shown). Under these conditions, the ligand produced a complex with the enzyme until the ligand concentration became higher than the concentration of active binding sites of the enzyme (Fig. 2).

In Fig. 2 the curve is characterized by the increasing of fluorescence intensity and displays a clearly pronounced kink. This kink is at the point corresponding to the ratio of 1 : 1 between the enzyme and the ligand concentrations. Because the kink in the linear region of the fluorescence intensity increase was recorded at the point corresponding to the ligand concentration equal to APE1 concentration, we concluded that 100% of APE1 molecules were active for binding. Thus, the decreased amplitude of the reaction burst in Fig. 1 could not be caused by a decrease in the concentration of active APE1 relative to the total concentration of the protein.

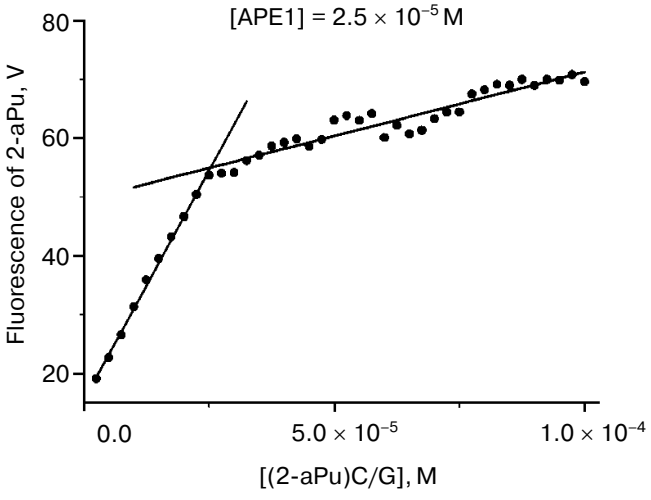


Fig. 2. Fluorescence titration of APE1 (25 μM) with undamaged 30-mer DNA duplex (2-aPu)C/G in NIR buffer. Experimental points are presented after the base line correction.

In work [24] pre-steady-state kinetics of the AP site cleavage by the APE1 were studied and, similarly to our case, it was shown that although the binding activity of the enzyme was at least 90%, the amplitude of the initial burst in the product accumulation was lower than the initial concentration of the enzyme. The authors explained that an inner equilibrium should exist between the enzyme–substrate complex and the enzyme–product complex due to significant rate of the reverse reaction relative to the rate of the forward reaction.

Based on our data, it was suggested that in the case of interaction of APE1 with DHU-containing substrate (Fig. 1), the amplitude of the burst in the kinetic curves of the product accumulation should be decreased due to existence of two conformations of the enzyme. Initially one part of the enzyme exists in conformation E1, which is energetically less advantageous but more active in the cleavage of DHU-substrate, whereas the another part of the enzyme exists in conformation E2, which is energetically more advantageous but significantly less active in the cleavage of the substrate. The less active form of the enzyme can either directly participate in formation of the initial enzyme–substrate complex or can be in equilibration with the more active form. Therefore, both the formation of the E2 complex with the substrate and the conformational transition of E2 into E1 are strongly shifted backward.

Thus, at the initial moment (Fig. 1, phase 1) at given APE1 and substrate concentrations only ~15% of the enzyme (form E1) produces the catalytically active enzyme–substrate complex. Therefore the accumulation of the product is limited by the rate of chemical cleavage of DHU-substrate by the fraction of APE1 existing in the more active conformation E1. During the second phase (Fig. 1) accumulation of the product of the DHU-substrate cleavage is limited either by the rate of formation of the E2 complex with DHU-substrate, or by the conformational conversion of E2 into E1. During the third phase (Fig. 1) the product accumulation is limited by the rate of dissociation of the enzyme–product complex. It seems that the enzyme release from the complex with the product is a limiting stage of the overall enzymatic process.

Kinetic curves of the 2-aPu fluorescence upon the interaction of APE1 with DHU-substrates. To determine the kinetic mechanism of the interaction of APE1 with substrate containing a DHU residue, we studied conformational changes in the DNA substrate by recording

changes in the intensity of fluorescence of a 2-aPu residue located in the substrate either from the 5′- or from the 3′-side of DHU.

On the binding of APE1 with an undamaged DNA lacking a DHU residue ((2-aPu)C/G) no changes in the 2-aPu fluorescence intensity were detected in the NIR and BER buffers (data not shown). However, on the interaction of APE1 with the substrate (2-aPu)DHU/G the changes in the intensities of 2-aPu fluorescence were observed under both BER and NIR conditions, and the shapes of the kinetic curves were similar (Fig. 3). In these curves three phases of fluorescence changes can be differentiated. During the initial rapid phase (<0.2 sec for APE1 in NIR; <1 sec for APE1 in BER) the fluorescence intensity of 2-aPu residues in both cases increased and reached an intermediate plateau. These initial ranges of increases in the fluorescence intensity of 2-aPu residues corresponded to the burst in Fig. 1 (phase 1), where the initial step in accumulation of the products of cleavage of ^{32}P-labeled DHU-containing substrate occurs, although we could not determine the amount of the product within such short time intervals. The second phase of the increase in the fluorescence intensity was observed up to ~1000 sec, and the third phase occurred at >1000 sec (Fig. 3) and corresponded to the third phase of the slow accumulation of products in Fig. 1.

In the case of the substrate (2-aPu)DHU/G that contains the 2-aPu residue on the 5′-side of DHU, the amplitude of the initial (<0.2 sec) increase in the 2-aPu fluorescence signal was extremely small in the NIR buffer (Fig. 3b). For the substrate DHU(2-aPu)/G with the 2-aPu residue located on the 3′-side of DHU, 2-aPu fluorescence signal was more pronounced on this time interval in NIR conditions (Fig. 4). In the case of the DHU(2-aPu)/G substrate, after a small increase with the reaching of an intermediate plateau (<0.2 sec, phase 1) (Fig. 4b) the fluorescence intensity decreased to about the initial value within a time interval of <10 sec (phase 2) (Fig. 4c). Then during the time interval of <1000 sec the fluorescence intensity slowly increased with reaching the new plateau (phase 3) (Fig. 4a). Until 7000 sec the fluorescence intensity did not change significantly. The increase of 2-aPu fluorescence signal within the first phase (Fig. 4b) seemed to correspond to the burst (phase 1) in Fig. 1 when the initial step of the accumulation of the cleavage products of the ^{32}P-labeled DHU-substrate occurred. During the second phase (Fig. 4c), the conformation of the cleavage product changed within the complex with APE1 leading to recovery of the 2-aPu stacking with the neighboring base, cytosine. During the third phase (Fig. 4a), the product conformation within the complex with APE1 changed once more, thus affecting the stacking of 2-aPu. This resulted in formation of a stable complex of the enzyme with the product, the concentration of which reached equilibrium condition during the time interval <1000 sec. The third phase of changes in the fluorescence intensity in Fig. 4a corresponded to the second phase in Fig. 3b. The further slow accumulation of products of the substrate DHU(2-aPu)/G cleavage by the enzyme was not accompanied by an increase in 2-aPu fluorescence, whereas upon the cleavage of other substrate (2-aPu)DHU/G the 2-aPu fluorescence increased also after 1000 sec. Thus, in the cleavage products released from the stable complex with APE1 in the case of the DHU(2-aPu)/G substrate

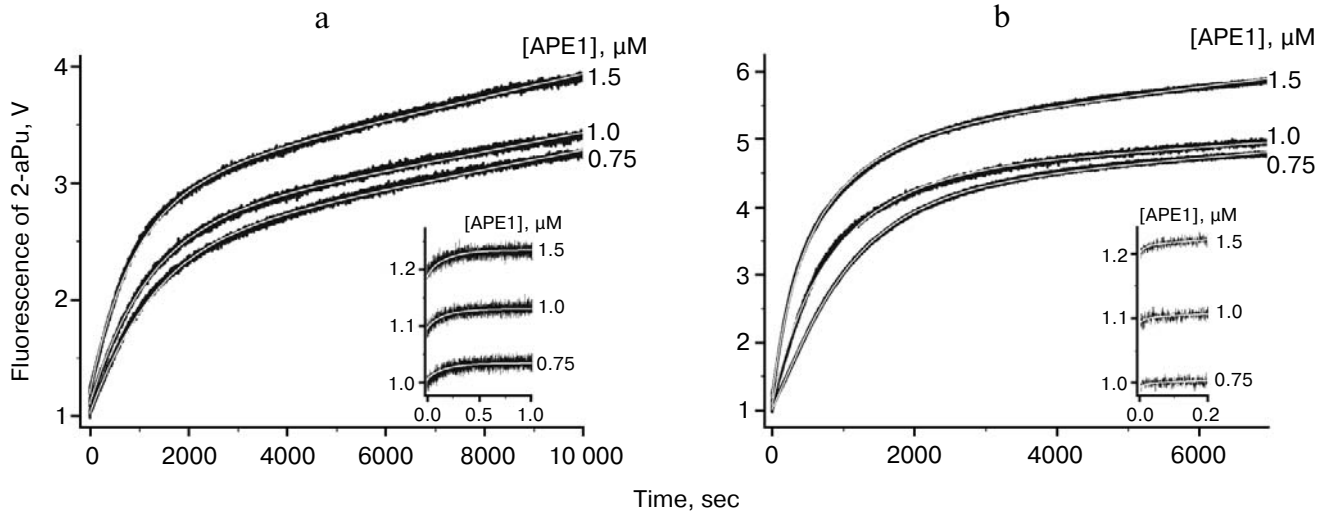


Fig. 3. 2-aPu fluorescence traces of the substrate (2-aPu)DHU/G (1 μM) upon interaction with APE1 in BER (a) and NIR (b) buffers. The solid white curves are theoretical approximations. The enzyme concentrations are shown on the right axis. Phase 1 presents the initial (for APE1 in NIR <0.2 sec; for APE1 in BER <1 sec) increase in the intensity of 2-aPu fluorescence with reaching the intermediate plateau. Phase 2 shows a subsequent increase in the fluorescence intensity at times to ~1000 sec. Phase 3 shows a slow increase in the fluorescence intensity at times >1000 sec.

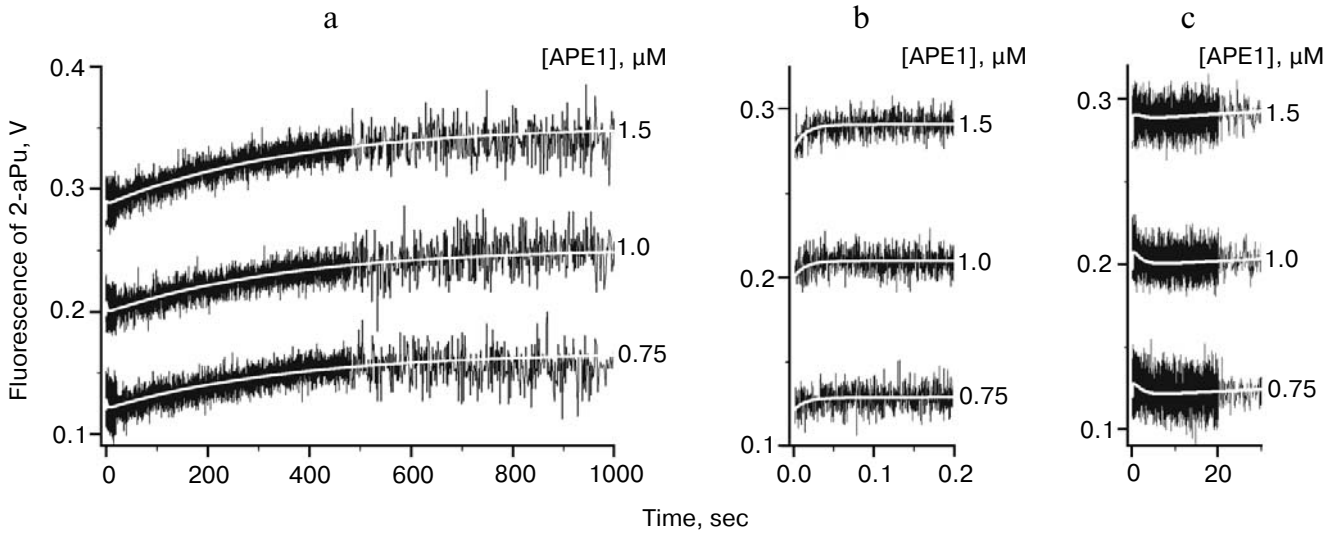


Fig. 4. 2-aPu fluorescence traces of substrate DHU(2-aPu)/G (1 μM) upon interaction with APE1 in the NIR buffer. The solid white curves are theoretical approximations. The enzyme concentrations are shown on the right axis. a) Total kinetic curve; b, c) initial regions of the kinetic curve presented with the expanded scale. Phase 1 corresponds to the initial increase in the 2-aPu fluorescence with the formation of intermediate plateau (b). Phase 2 corresponds to a decrease in the fluorescence extending to ~10 sec (c). Phase 3 corresponds to an increase in the fluorescence intensity extending to 1000 sec (a).

the 2-aPu stacking was not affected, whereas it was disturbed in the case of (2-aPu)DHU/G. It seems that disturbance of the 2-aPu stacking in the cleavage product of the (2-aPu)DHU/G substrate was caused by dissociation of a single-stranded 10-meric oligonucleotide containing 2-aPu from the duplex product. In the case of DHU(2-aPu)/G the 2-aPu residue, upon the substrate cleavage by the enzyme, was located in a 20-meric oligonucleotide fragment that did not dissociate from the duplex.

In the present work conformational changes in APE1 during its interactions with the substrate DHU/G were studied in more detail in comparison with our previous work [14]. The initial decrease in the APE1 Trp fluorescence on the kinetic curves in the BER buffer seemed to correspond to formation of the complex of more active enzyme conformation E1 with the substrate and, possibly, to isomerization of the enzyme–substrate complex. Then the intensity of the protein fluorescence slightly increased (Fig. 5) with the reaching plateau at time intervals of >50 msec synchronously with the initial increase of 2-aPu fluorescence signal for the substrate (2-aPu)DHU/G (Fig. 3a, phase 1), i.e. it corresponded to the first rapid stage of the substrate cleavage. Trp fluorescence traces of APE1 upon the interaction with substrate DHU/G in the NIR buffer were previously described in our work [14] as two sequential equilibrium stages occurring before the cleavage of DNA. The results of our present study (Figs. 3b and 4) give evidence that the first equilibrium stage can really correspond to formation of the enzyme E1 complex with the substrate. The second stage corresponds to changes in the protein conformation within the complex with the product of the enzymatic reaction already after the catalytic stage. This second equilibrium stage recorded by changes in the intensity of tryptophan fluorescence [14] occurs simultaneously with changes in the conformation of the product of DHU(2-aPu)/G cleavage within the complex with APE1, which follows the substrate cleavage and results in recovery of the 2-aPu stacking (Fig. 4c).

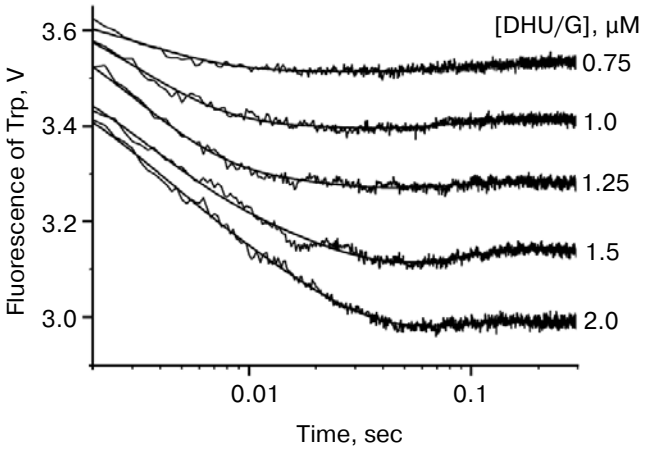


Fig. 5. Trp fluorescence traces of APE1 (1 μM) upon interaction with the substrate DHU/G in BER buffer. Solid curves are the result of fitting procedure. The DHU/G concentrations are shown on the right axis.

Kinetic scheme of APE1 mechanism for nucleotide incision repair pathway. Based on our findings, it was suggested that APE1 should exist in two conformations. At the initial moment a part of the enzyme exists in conformation E1, which is less advantageous energetically but

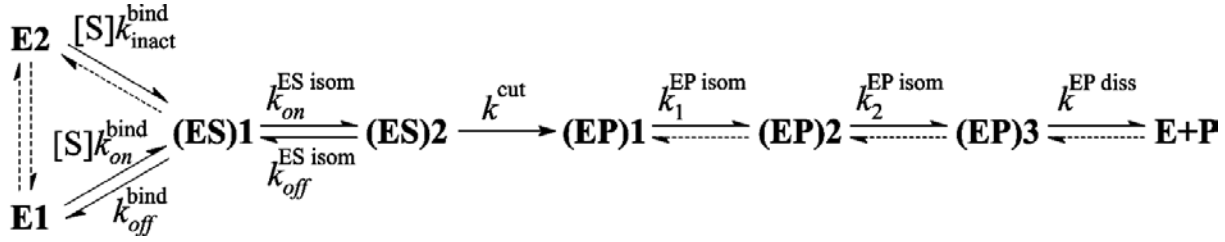
more active in the cleavage of the DHU-substrate. The another part of the enzyme exists in conformation E2, which is more advantageous energetically but significantly less active in the catalytic cleavage of the DHU-substrate. The less active form of the enzyme can either slowly produce the initial enzyme–substrate complex or slowly transform into the more active form.

Trp fluorescence traces of APE1 upon interaction with the DHU/G substrate in BER buffer (Fig. 5) reveal three stages which can be attributed to the initial formation of the complex of the enzyme in the more active conformation E1 with the DHU-substrate, the isomerization of this complex, and the stage of chemical cleavage of the substrate (Scheme). Forward and reverse rate constants of formation of the initial enzyme–substrate complex (k_{on}^{bind}, k_{off}^{bind}) and of its isomerization ($k_{on}^{ES\ isom}$, $k_{off}^{ES\ isom}$), as well as the rate constant of the substrate cleavage (k^{cut}) (Scheme) are presented in the table.

On the kinetic curves corresponding to accumulation of products during the interaction of APE1 with the ^{32}P-labeled substrates DHU/G in the BER (Fig. 1a) and NIR (Fig. 1b) buffers and (2-aPu)DHU/G in the NIR buffer (data not shown) three kinetic stages were differentiated. The first rapid stage corresponded to the chemical cleavage of the DHU-substrate by APE1 in the active conformation E1 (Fig. 1, phase 1). The slower second stage was limited either by the rate of the direct formation of the complex of the less active enzyme form E2 with the DHU substrate or by the rate of conformational transition of E2 into E1 (Fig. 1, phase 2). The slowest third stage we have ascribed to the process of the enzyme release from the complex with the product of the substrate cleavage (Fig. 1, phase 3). This last stage seems to be limiting of the overall enzymatic process and determining the reaction rate in the steady-state conditions. The data presented in Fig. 1 do not allow us to obtain exact kinetic parameters of the first stage of accumulation of products of DNA cleavage (the burst stage). Rate constants of the second (k_{inact}^{bind}) and third stages ($k^{EP\ diss}$) are presented in the table.

Three phases can be also described on the kinetic curves of the interaction of APE1 with the substrate (2-aPu)DHU/G recorded by changes in the 2-aPu fluorescence signal in the both BER and NIR buffers (Fig. 3). As before, the first phase is suggested to correspond to a rapid chemical cleavage of the substrate by the enzyme (Fig. 3, phase 1). The values of rate constants corresponding to this phase (k^{cut}) for the APE1 interaction with the substrate (2-aPu)DHU/G (recording of the 2-aPu fluorescence) and with the substrate DHU/G (recording of Trp fluorescence) in the BER buffer were similar (see table). Rate constants corresponding to the second phase ($k_2^{EP\ isom}$) for the interaction of APE1 with the substrate (2-aPu)DHU/G were lower than the rate constants of the second stage (k_{inact}^{bind}) isolated on the kinetic curves of accumulation of ^{32}P-labeled products (Fig. 1) in the corresponding buffers (the table). Therefore, we supposed that the second stage in Fig. 3 should be limited by a slow conformational change of the APE1 complex with the product of the DHU-substrate cleavage ($k_2^{EP\ isom}$) but not by the process limiting the second stage of accumulation of the product of the ^{32}P-labeled DHU-substrate cleavage by APE1 (Fig. 1), i.e. by reactions with involvement of the inactive form of the enzyme (k_{inact}^{bind}). The third phase isolated on the kinetic curves of the interaction of APE1 with the substrate (2-aPu)DHU/G (Fig. 3) we assigned to the enzyme release from the stable complex with the product. Rate constants of the limiting stage of the enzyme release from the complex with the product ($k^{EP\ diss}$) obtained by fitting procedure from 2-aPu fluorescence traces of APE1 interaction with the substrate (2-aPu)DHU/G were in a good agreement with the values of corresponding constants obtained from the kinetic curves of accumulation of ^{32}P-labeled products (the table).

On 2-aPu fluorescence traces detected for the interaction of APE1 with the DHU(2-aPu)/G substrate recorded in the NIR buffer (Fig. 4) three phases were also discerned. The first phase, as previously, was considered to correspond to the rapid chemical cleavage of the substrate by the enzyme (Fig. 4b, phase 1). Rate constants of the catalytic stage of the cleavage (k^{cut}) by APE1 of the substrates DHU(2-aPu)/G and (2-aPu)DHU/G in the NIR buffer were nearly equal (the table). The second phase corresponded to the stage of the conformational transition of the enzyme within the complex with the product of the enzymatic reaction (Fig. 4c, phase 2). The rate constant of this conformational transition ($k_1^{EP\ isom}$) is presented in the table. The third phase for the substrate DHU(2-aPu)/G in the NIR buffer (Fig. 4a, phase 3) was concurrent with the second phase for the substrate (2-aPu)DHU/G (Fig. 3b, phase 2) and represented a slow conformational change of the APE1 complex with the enzymatic reaction product. Rate constants ($k_2^{EP\ isom}$) of



Rate constants of the interaction of APE1 with DHU-containing substrates in BER and NIR buffers

	DHU/G (from data of Fig. 5)	[^{32}P]**DHU/G**[a,c] (from data of Fig. 1a)	**(2-aPu)DHU/G**[a,c] (from data of Fig. 3a)
BER buffer			
k_{on}^{bind} ($M^{-1}\cdot sec^{-1}$)	$(3.8 \pm 0.01) \times 10^7$		
k_{off}^{bind} (sec^{-1})	131 ± 0.2		
$k_{on}^{ES\ isom}$ (sec^{-1})	145 ± 0.6		
$k_{off}^{ES\ isom}$ (sec^{-1})	36 ± 0.2		
k^{cut} (sec^{-1})	20 ± 0.1	not determined	15 ± 0.7
k_{inact}^{bind} (sec^{-1})		$(3.2 \pm 0.3) \times 10^{-3}$	
$k_2^{EP\ isom}$ (sec^{-1})			$(1.6 \pm 0.06) \times 10^{-3}$
$k^{EP\ diss}$ (sec^{-1})[d]		$(2.2 \pm 0.2) \times 10^{-5}$	$(1.8 \pm 0.02) \times 10^{-5}$
NIR buffer			
	[^{32}P]**DHU/G**[b,c] (from data of Fig. 1b) {[^{32}P]**(2-aPu)DHU/G**}[b,c]	**(2-aPu)DHU/G**[b,c] (from data of Fig. 3b)	**DHU(2-aPu)/G**[b,c] (from data of Fig. 4)
k^{cut} (sec^{-1})	not determined	49 ± 10	53 ± 4
k_{inact}^{bind} (sec^{-1})	$(1.6 \pm 0.2) \times 10^{-2}$ {$(2.5 \pm 0.2) \times 10^{-2}$}		
$k_1^{EP\ isom}$ (sec^{-1})			0.56 ± 0.1
$k_2^{EP\ isom}$ (sec^{-1})		$(3.7 \pm 0.03) \times 10^{-3}$	$(2.7 \pm 0.1) \times 10^{-3}$
$k^{EP\ diss}$ (sec^{-1})[d]	$(1.3 \pm 0.3) \times 10^{-5}$ {$(2.2 \pm 0.3) \times 10^{-5}$}	$(1.5 \pm 0.01) \times 10^{-5}$	

[a] Rate constants (k_{on}^{bind}, k_{off}^{bind}, $k_{on}^{ES\ isom}$, $k_{off}^{ES\ isom}$) of the enzyme–substrate complex formation are taken from data of Fig. 5.
[b] Rate constants ($k_{on}^{bind} = 2.9\cdot10^6$ $M^{-1}\cdot sec^{-1}$, $k_{off}^{bind} = 18$ sec^{-1}) of the enzyme–substrate complex formation are taken from work [14].
[c] Concentration of enzyme involved in the substrate cleavage was 15% of initial concentration of APE1.
[d] The errors in $k^{EP\ diss}$ include the contribution of APE1 inactivation due to long-time incubation.

this slow conformational change in the enzyme complex with products of the enzymatic cleavage of the substrates (2-aPu)DHU/G and DHU(2-aPu)/G in the NIR buffer were similar in values (the table).

We have estimated the value of the equilibrium dissociation constant K_d of the enzyme–substrate complex by titrating APE1 with the substrate DHU/G under steady-state conditions in the NIR buffer (data not shown). The value $K_d = (1.3 \pm 0.03)\cdot10^{-6}$ M was obtained, which virtually coincided with the value of $K_d = 1.4$ μM for the enzyme–product complex [14]. Consequently, the extremely slow release of APE1 from the stable complex with the product of the cleavage cannot be explained by simple equilibrium dissociation. However, we have not detected formation of Schiff bases when treating with $NaBH_4$ of the reaction mixture of APE1 with DHU/G 8 and 30 min and 5 and 24 h after the mixing of the enzyme with the substrate under NIR conditions (data not shown). The mechanism of APE1 release from the complex with the product remains unclear.

Based on obtained data, a kinetic scheme of the enzymatic process was proposed describing the mechanism of conversion of the DHU-containing substrate by APE1 during nucleotide incision repair (the Scheme). At the initial moment one part of the enzyme has the conformation more active for cleavage of the DHU-substrate, and the another part of the enzyme has the less active conformation. The less active form of the enzyme can either participate in formation of the initial enzyme–substrate complex or be in slow equilibrium with

the more active form. The more active conformation of the enzyme rapidly forms the initial enzyme–substrate complex, which is first isomerized. Then the 5′-phosphodiester bond of the substrate is rapidly hydrolyzed, and the complex of the enzyme with the enzymatic reaction product undergoes two stages of conformational changes. Afterwards the enzyme release from the stable complex with the product is a limiting stage of the process.

Specificity of APE1 interaction with DHU-containing substrate. The present work has shown that the rate of hydrolytic cleavage of DHU-substrate by the APE1 in NIR pathway is comparable with the rate of cleavage of DNA substrate containing apurinic/apyrimidinic site in BER pathway. Rate constants of catalytic cleavage by APE1 of DHU-substrates in the NIR buffer (k^{cut} = 49-53 sec^{-1}; table) optimal for cleavage of DHU were only slightly lower (~1.3-1.4-fold) than rate constants of the catalytic cleavage of F-containing substrate (k^{cut} = 68 sec^{-1}) and nearly twofold lower than the corresponding constant for AP-substrate (k^{cut} = 97 sec^{-1}) in the BER buffer optimal for cleavage of AP and F [14]. In the BER buffer rate constants of catalytic cleavage of DHU-substrates (k^{cut} = 15-20 sec^{-1}; table) were ~15-20-fold higher than the corresponding constant for the F-containing substrate in the NIR buffer (k^{cut} = 1 sec^{-1}) [14]. The data suggest that the reaction with such oxidative DNA damage as DHU catalyzed by APE1 without involvement of DNA glycosylases is very rapid. Thus, the involvement of the APE1 in the NIR process can be biologically relevant.

It is known that APE1 bound to damaged DNA promotes binding of polymerase β with formation of a triple complex [25]. Moreover, APE1 interacts with flap endonuclease 1 (FEN 1) and with PCNA [26]. The presence of APE1 also stimulates the flap-structure specific endonuclease activity of FEN 1 [26, 27]. APE1 is responsible for the efficient repair even in the absence of PCNA [27]. In addition, APE1 enhances a successive binding and catalysis by DNA polymerase δ and DNA ligase I [28]. Under excess of the enzymes (APE1 and polβ) relative to the DNA substrate, APE1 promotes DNA synthesis associated with the strand-displacement catalyzed by DNA polymerase β [29]. It seems that the existence of a very tight complex between APE1 and the product of the DHU-substrate cleavage is favorable for coordinated binding of other proteins involved in repair to the substrate and for transfer of the substrate from one enzyme to another.

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