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REVIEW

Human Deoxyribonucleases

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Abstract—Although mammalian deoxyribonucleases were discovered more than 60 years ago, interest in these enzymes is not weakening. During the last decade, intensive studies of human DNases culminated in discovery of several novel enzymes exhibiting DNase activity. These include an unusual DNase, lactoferrin. For some enzymes, their three-dimensional structure and molecular mechanisms underlying their functioning have been elucidated. In patients with some autoimmune and viral diseases, catalytic antibodies also contribute to alternative pathways of DNA hydrolysis. Some enzymes exhibiting DNase activity play an important role in pathogenesis of various diseases and also in programmed cell death (apoptosis). This review highlights recent achievement in human deoxyribonuclease research. It also considers mechanisms of DNA hydrolysis. The review also summarizes modern data on the biological role of these enzymes in functioning of the human organism, realization of its protective mechanisms, and possible applications of DNases in medicine.

Key words: human, deoxyribonuclease, structure, action mechanism, DNA-hydrolyzing antibodies

Enzymatic cleavage of nucleic acids was originally described in 1903 [1]. In 1940, based on the sugar specificity of nucleases, Kunitz subdivided the nucleic acid hydrolyzing enzymes into two groups: ribonucleases and deoxyribonucleases. A unique property of deoxyribonucleases (DNases) consists in the fact that they effectively hydrolyze the phosphodiester bond, the most stable chemical bond found in biological molecules [2]. DNases play an important role in metabolism of nucleic acids and maintenance of physiological DNA concentration in the human body [1]. For example, increase of DNA concentration in blood may result in accumulation of DNA–protein complexes, induction and elaboration of anti-DNA antibodies, and development of various autoimmune diseases (see for review [3-5]). DNases also play a certain role in protection of organisms against xenobiotic nucleic acids (see for review [1, 6, 7]).

The list of the most studied human DNases includes nonspecific DNase I and DNase II and also phosphodiesterase I (PDE) effectively hydrolyzing DNA and RNA. It should be noted that structure and functioning of mammalian DNase I were reviewed more than 20 years ago (in 1981) [8]. The "latest" reviews on mammalian DNase II were published in the 1970s [9, 10]. Enzymatic properties of the family of mammalian PDE type I and their biological properties were summarized in 1977 [9], and physicochemical characteristics of these enzymes were reviewed in 1998 [6]. Properties of human DNases have not been summarized at all.

Recently, new data on structure, action mechanism, and biological properties of DNase I, DNase II, and PDE I have accumulated and new enzymes involved in degradation of cellular DNA have been discovered. It was also shown that lactoferrin is a major milk DNase of lactating women, and properties of this enzyme differ from those found in DNases [11]. During the last decade, the existence of catalytic antibodies exhibiting DNase activity was firmly recognized in blood of patients with certain autoimmune and viral diseases. The list of DNA hydrolyzing enzymes is constantly increasing and new data on involvement of these enzymes into the main biological processes in cells appear. Several studies demonstrated that DNases and DNA-hydrolyzing antibodies can be successfully employed for both diagnostics and medical treatment of some diseases. The present review summarizes recent data on structure, function, biological role, and promise for use in medicine of various human DNA hydrolyzing enzymes.

Abbreviations: bDNase) bovine DNase; hDNase) human DNase; rhDNase) recombinant human DNase; PDE) phosphodiesterase.

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DNase I

This is one of the best-studied enzymes with known active site structure and mechanism of DNA hydrolysis. Human DNase I (hDNase I) is a member of the mammalian DNase I family. It is characterized by neutral pH optimum, bivalent metal ion requirement for catalytic activity, and formation of oligonucleotides with phosphate at 5′-end (EC 3.1.21.1) [8, 9].

Studies of human neutral deoxyribonuclease started in the 1950s. Subsequently, enzyme preparations from pancreas [12, 13], kidneys [14], urine [15, 16], blood [13], and seminal fluid [17] were isolated and characterized. Comparative studies revealed that human DNase I preparations isolated from various organs and body fluids share similar physicochemical [18] and catalytic properties [13]. Some differences were found in composition of carbohydrate component, reflecting tissue specificity of glycosylation [17].

Characterization of the DNase I gene and the level of its expression in the body. DNase I is encoded by a single gene (*DNASE1*), located on chromosome 16 within the p13.3 region [19]. This gene of 3200 base pairs (bp) consists of nine exons and eight introns [20]. Six allele variants of *DNASE1* characterized by point nucleotide substitutions in the coding region have been described [20-24]. *DNASE1*2* encodes a protein in which Gln222 is substituted for Arg; this determines classic polymorphism of DNase I recognized by isoelectric focusing [20]. Using the suggestion that *DNASE1*1* is an initial gene (Gln222 is highly conservative in other mammalian species) Yasuda et al. proposed the following phylogenic scheme of hDNase I (Fig. 1) [24]. All found mutations (except *DNASE1*6*) insignificantly change catalytic characteristics and antigenic properties. Allele variants (*DNASE1*5* and *DNASE1*6*) are very rare and for four others the following distribution frequencies were found in a Japanese population: *DNASE1*1*, 0.5503; *DNASE1*2*, 0.4369; *DNASE1*3*, 0.0116; *DNASE1*4*, 0.0012 [25].

DNase I is a secretory protein; this is confirmed by the presence of a hydrophobic signal peptide at the N-terminus and high glycosylation level [26, 27]. Its cell localization corresponds to the traditional secretory route: rough endoplasmic reticulum, Golgi apparatus, and secretory granules. Distribution of hDNase I in the body may be defined as the pancreatic type, because specific activity of hDNase I [28] and level of its expression [27] in human organs decrease in the following order: pancreas > kidneys > small intestine > liver, stomach, large intestine, thymus, etc. Among extracellular liquids significant level of neutral DNase was found in urine (~200 ng/ml [29]), whereas its level in blood was only 3-10 ng/ml [29, 30] and the proportion of active form was even less due to the presence of a natural inhibitor of this enzyme, actin (see below). A significant portion of serum DNase I has pancreatic origin, and the level of the serum enzyme activity correlates with the state of the pancreas [13, 31]. Other sources of blood neutral DNase include anterior and

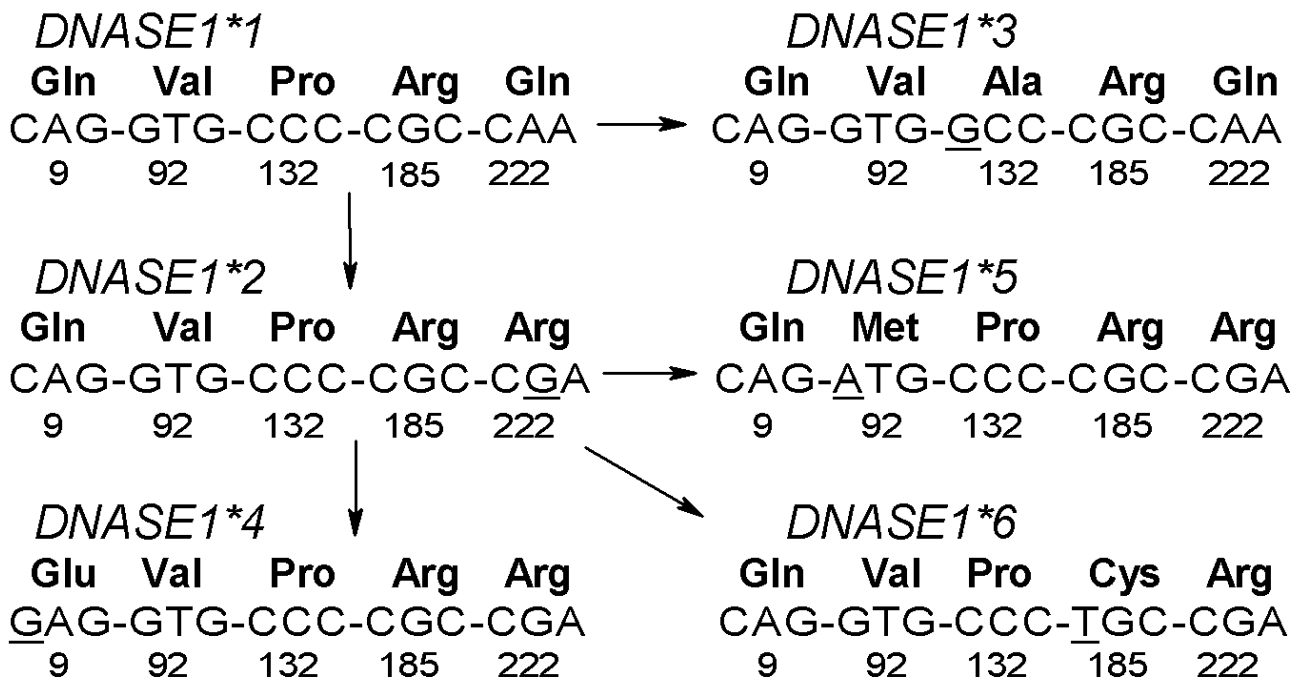

Fig. 1. Molecular-genetic basis for human DNase I polymorphism [26]. Numbers indicate amino acid residues from N-end of mature protein. Sites of substitutions in the nucleotide sequence are underlined.

intermediate pituitary, and its secretion is influenced by hypothalamic hormones [32].

Structure and physicochemical characteristics. DNase I is a monomeric protein that consists of 260 residues; its calculated molecular mass is 30 kD [26]. The amino acid sequences of bovine and human DNases I share 78% identity; the major differences are in the hydrophilic region at the protein surface. According to X-ray analysis [33, 34], this enzyme belongs to α,β-proteins with approximate 3D sizes of 45 × 40 × 35 Å. A large hydrophobic protein core, formed by 30% of all residues, consists of two tightly packed β-sheets; eight α-helices and several loops are located on the periphery. This backbone is mainly responsible for the structural stability and rigidity of DNase I. Protein structure is additionally stabilized by two disulfide bonds (C101–C104, C173–C209) and two high affinity Ca^{2+}-binding sites. DNase I contains two structurally similar domains formed by residues 1-123 and 123-260, respectively. These domains are well superimposed; this suggests duplication of an ancestor precursor gene during phylogeny [34].

Neutral DNase I is an acidic glycoprotein with total carbohydrate component of 11.8% [16, 26, 35]. Both glycoside residues (at Asn18 and Asn106) contain N-acetylglucosamine, sialic acid, and significant amount of mannose; one fucose molecule is contained in the oligosaccharide chain attached to Asn106 [35]. It was recently found that carbohydrate residues of hDNase I may be phosphorylated [35]. Only three non-lysosomal proteins containing mannose-6-phosphate in their oligosaccharide component are known to date; however, the biological importance of this phosphorylation remains unclear. Perhaps, it might indicate potential involvement of lysosomes in their catabolism. Polymorphism of DNase I by isoelectric points (p*I* within the pH range 3.5-4.3 [13]) is usually explained by existence of several co-dominant alleles of the DNase I gene and also by different content of sialic acid and mannose-6-phosphate in the carbohydrate component of this enzyme [35, 36].

Active site structure and mechanism of hydrolysis. According to X-ray analysis data, the active site topography of hDNase I is almost identical to that of bovine DNase I (bDNase I); amino acid residues involved in binding and hydrolysis of DNA are also highly conservative [37]. DNase I interacts only with the minor groove of B-forms of DNA; this groove is fully filled with the side chain of Arg41 and the exposed loop with Tyr76, whereas positively charged and hydrophilic amino acid residues form contacts with several phosphates of both strands; the latter bonding involves six base pairs [38, 39]. Unusual hydrophobic contact of Tyr76 with deoxyribose results in inversed configuration at the attacked phosphorus atom, and this causes conformational changes in the DNA molecule: the minor groove enlarges by 3 Å, whereas the double helix bends by 20° towards the major groove. Tyr76 and Arg41 are required for binding of the enzyme to DNA and also for the correct orientation of the attacked phosphodiester bond [40]. Arg41 is the only residue interacting with DNA bases. It interacts with O2- or N3-atoms of pyrimidine or purine bases (at positions 3 and 4 to the 5′-end of the cleavage site), respectively, in the DNA strand that is complementary to the excised one. Contacts between the enzyme and sugar-phosphate backbone of DNA are mainly stabilized by hydrogen bonds; an ionic bond is formed only between Arg111 and the phosphate adjacent to the cleavage group.

Structural aspects of recognition and DNA hydrolysis by DNase I were summarized by Suck in 1994 [41]. DNA cleavage occurs via the acid–base catalytic mechanism, which involves two histidine residues: His134 acts as a base accepting a water proton, whereas His252 acts as an acid, which gives a proton to the leaving O3′-group [42]. An activated water molecule acts as a nucleophile attacking the phosphorus atom (S_N2 mechanism) and cleaves the P–O3′-bond (Fig. 2). (Electrophilicity of this phosphorus atom is increased by a Mg^{2+} ion interacting with the oxygen atom of the phosphate group.)

Catalytic properties of DNase I. Neutral deoxyribonuclease is 100-500 times more active in hydrolysis of double strand sites of DNA than of single strand; this is attributed to the interaction of the enzyme with both strands of a substrate molecule [43]. DNase I does not exhibit any preference towards certain bases of nucleotide sequences; however, it is sensitive to conformations of the double helix [41, 44]. The rate of hydrolysis strongly depends on such parameters of double helix as width and depth of minor grove and also on rigidity of its structure. B-form of DNA of mixed composition is the best substrate for DNase I, whereas extended A–T or G–C sites are relatively resistant to hydrolysis.

Significant differences (up to two orders of magnitude) in efficacy of cleavage of phosphodiester bond between neighboring bases may be explained by local influences of double helix such as helix turn angle and orientation of the attacked phosphate group [45]. The interaction of Arg41 with two bases of the complementary strand makes an important contribution to the specificity of hydrolysis [40]. Studying hydrolysis of a DNA fragment of 160 bp, it was found that DNase I was sensitive to the presence of an A–T pair at the third position from the site of hydrolysis and the following nucleotide sequence 5′-(A/T)Py(A/T)↓(A/T)(A/G/C)-3′ was the preferential environment for the cleavage bond [46]. Due to a characteristic feature of minor grove structure, four phosphodiester bonds were completely resistant to proteolysis at the 5′-end [43, 47]. Bivalent metal ions are also crucial for specificity of hydrolysis [48]; they may influence DNA conformation and mechanism of DNase action (see below). For example, Cu^{2+} and Hg^{2+} exhibit high affinity to guanine and thymine, respectively, and protect these sites against attack by DNase [49].

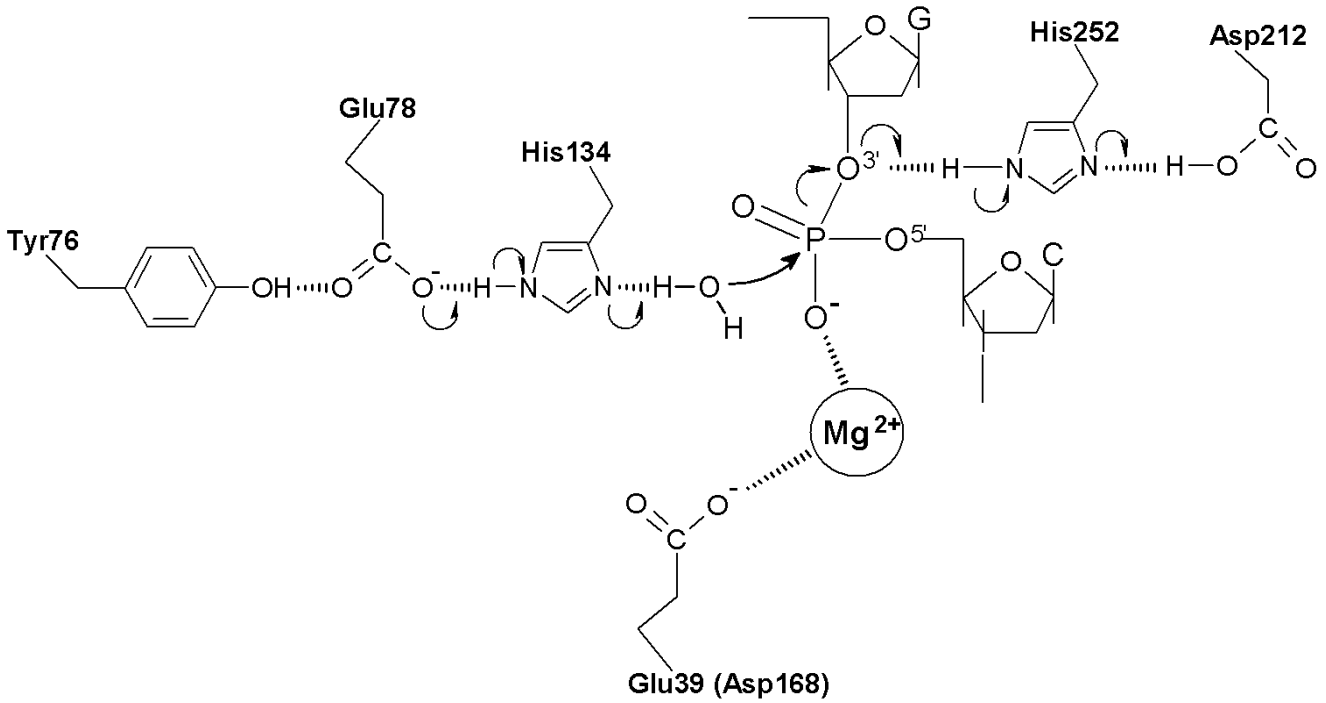

Fig. 2. Mechanism of acid–base catalysis by DNase I during phosphodiester bond cleavage (according to [49, 50] with modifications).

DNase I belongs to the class of Mg^{2+}+Ca^{2+}-dependent nucleases by dependence of hydrolytic activity on the presence of bivalent metals. Optimal concentrations of Mg^{2+} and Ca^{2+} for manifestation of catalytic activity are 10 and 1 mM, respectively [13]. Magnesium ion is involved in electrophilic catalysis of the phosphodiester bond cleavage, whereas Ca^{2+} maintains optimal enzyme conformation [41]. Binding of one or two Mg^{2+} ions in the active site involves Glu39 and/or Asp168 [42, 50]. This low affinity site may bind other metal ions with various efficacy [51]. On the surface of DNase I there are two high affinity binding sites (with K_d values of 10^{-5} M) for Ca^{2+} [52, 53]. The first Ca^{2+}-binding site is formed by Asp201 and Thr203 [54] (or by Thr207 [55]). Ca^{2+} binding at this site stabilizes the loop formed by Asp201-Thr207; the latter is crucial for protection of the disulfide bridge (C173–209) against reduction [13] and for resistance against serine proteases, which may potentially attack the region Ser174-Ser179 [56]. The second Ca^{2+} binding site is formed by Asp99, Asp107, and Glu112; it stabilizes the loop Asp99-Asp107. The latter influences conformation of the adjacent loop 73-76, which binds to the minor grove of the substrate molecule [54]. Increase in the rate of DNA hydrolysis in the presence of $CaCl_2$ is mainly determined by increased DNase affinity for substrate, whereas V_{max} remains unchanged [57]. Tighter enzyme–DNA interaction increases processivity of hydrolysis and the proportion of double strand breaks in the substrate [13]. Interestingly, an increased proportion of positively charged amino acids on the hDNase I surface forming additional contacts with the substrate molecule significantly reduced K_m value and increased processivity of hydrolysis; under these conditions the requirement of the enzyme for Ca^{2+} is significantly reduced [37].

In the presence of 2.5 mM $MgCl_2$, Sr^{2+} and Ba^{2+} can effectively replace Ca^{2+}; however, they poorly catalyze DNA hydrolysis [57]. In the absence of other metal ions, transition metal ions Mn^{2+} and Co^{2+} exhibit high attacking ability and the proportion of double strand breaks was markedly higher than in the case of Mg^{2+} + Ca^{2+} [58]. This effect especially pronounced in the presence of Mn^{2+} and low ionic strength of the medium may be attributed to formation of chelates between the N7-atom of a guanine moiety and phosphate; this unwinds and destabilizes the double strand, decreases melting temperature, and therefore promotes DNA attack by the enzyme [59]. Monovalent cations such as Na^+, K^+, and NH_4^+ cause nonspecific inhibition of DNase I [58, 60, 61]. Since monovalent cations also decrease processivity of hydrolysis, K_i values determined by various methods significantly differ. The IC_{50} value (concentration required for 50% inhibition of enzyme activity) for NaCl was about 60 mM when catalytic activity was evaluated by the hyperchromatic effect [37], and the IC_{50} value was 150 mM when assay of reaction rate was registered by disappearance of supercoiled DNA form [13].

Under optimal reaction conditions (the presence of Mg^{2+} and Ca^{2+} or Mn^{2+}, low ionic strength, pH 7.0)

DNase I introduces from one to several single strand breaks into both strands, which are slightly shifted by several nucleotides [62]. The low rate of double strand break formation (<50% under any reaction conditions) is positively influenced by high temperature, low ionic strength, and the presence of negative supercoils [58]. The K_m value for rhDNase I obtained during hydrolysis of supercoiled pBR322 plasmid under low ionic strength conditions was 5.7 ± 0.8 nM or 16 ± 2 μg/ml; this value corresponds to 50 μM (if recalculated per nucleotide number) [61]. Significantly higher K_m values (about 229 ± 33 μg/ml) were obtained during kinetic study of calf thymus DNA hydrolysis evaluated by the hyperchromatic effect in the presence of 150 mM NaCl.

Stability of DNase I. In contrast to bovine DNase I and the enzyme from other animals, neutral hDNase I is sensitive to acidification of the medium [28]. Incubation of this enzyme at pH 4.0 for 10 min reduced its activity by 75%, whereas the same incubation at pH 3.0 completely abolished catalytic activity. Heating at 67°C causes irreversible denaturation of DNase I, which is accompanied by unfolding of the protein globule and aggregate formation [63]. Disaccharides and Ca^{2+} stabilize the enzyme against heat denaturation, whereas other bivalent metal ions (Mg^{2+}, Mn^{2+}, Zn^{2+}), urea, and guanidinium chloride accelerate this process. Incubation of neutral DNase in a neutral buffer with EDTA reduced the enzyme activity; this was due to deamination of Asn74 and aggregate formation, which was caused by breakage of the S–S-bridge at 173-206 and formation of intermolecular bonds [64, 65].

Actin is a natural DNase I inhibitor. Almost all mammalian tissues contain a specific protein inhibitor of DNase I that represents up to 5-10% of total soluble protein content [9]. This inhibitor of 43 kD was able to form polymeric high molecular weight aggregate. It was identified as the known structural protein, actin. Only monomeric actin (G-actin) effectively inhibits DNase I (K_i = 1.3 nM) by forming a stoichiometric (1 : 1) complex. The actin-binding site is located far from the catalytic site, and inhibition of DNase I by actin is determined by appearance of steric difficulties during the interaction of the enzyme with DNA. The interaction of DNase I with actin involves hydrophobic, electrostatic, and hydrogen bonds. Site-directed mutagenesis experiments revealed the ultimate role of Tyr65 and Ala114 of DNase I for interaction with actin [66]. Detailed study revealed that Ca^{2+} and ATP (containing specific binding sites on actin) influence complex formation between DNase I and actin [67]. Inhibition of DNase I activity by actin is a reversible process. DNase I was restored almost completely after heat denaturation of actin (10 min at 50°C). The biological importance of DNase I inhibition by actin remains unclear; however, this phenomenon may be potentially important in mitosis and apoptosis [68]. In human blood serum actin content may reach 100 μg/ml. Increase in actin content in blood was found in patients with systemic lupus erythematosus [69].

Medical application of DNase I. Many attempts have been made to use hDNase I in diagnostics and treatment of various diseases. Diagnostic approaches are usually based on analysis of DNase activity in body fluids (mainly blood) [70, 71] or on phenotype determination by isoelectric focusing for separation of enzyme isoforms [72]. For example, in a Japanese population a significant deficit in *DNASE1*1-2* phenotype was found in patients with impairments in gastrointestinal tract functioning [73]. There were correlations between blood endonuclease activity and some pathological states. For example, significant increase in DNase activity in blood was found during development of breast cancer [74], whereas in patients with chronic pancreatitis [75], stomach cancer [76], and glomerulonephritis [77, 78] blood activity of this enzyme was below normal values. Intravenous or subcutaneous administration of rhDNase I did not influence any marker in patients with systemic lupus erythematosus [79]. Recently, rhDNase I has been employed for improvement of respiratory tract functioning in patients with cystic fibrosis; the positive effect is due to hydrolysis of high molecular weight DNA and a decrease in viscosity of mucosal secretions [80-83]. A mutant form of rhDNase I, insensitive to actin inhibition (and much more active in DNA hydrolysis than wild type DNases I), is more promising for use in medical practice [69].

Study of the action of rhDNase I on DNA in undiluted blood samples revealed that the enzyme concentration of 50-100 ng/ml is sufficient for effective removal of high molecular weight DNA from blood circulation. The presence of various ratios of DNA and DNA–protein complexes did not influence the rate of nucleoprotein complex degradation [84].

DNase I-like nucleases. At the end of the 1990s three new human nucleases—DNase γ, DNase X, and DNAS1L2—were discovered. They share similarity in amino acid sequence and catalytic properties with DNase I [85-88]. Amino acids involved in formation of catalytic and DNA- and calcium-binding centers are highly conservative. Genes encoding DNase I-like nucleases differ in chromosomal localization and expression level in various organs. DNase X is mainly expressed in skeletal muscles and myocardium [86, 88]. Human DNase γ is mainly distributed in macrophages, liver, spleen, and some other organs [89]; pronounced expression of *DNAS1L2* was found in brain, lung, and placenta [27, 85]. Some authors reported that DNase γ is a non-secretory protein localized in the perinuclear space [27], whereas others found its active secretion into the extracellular space [89]. DNase X is a non-secretory protein localized in cytoplasm, whereas cell localization of DNAS1L2 still requires clear evidence [90, 91].

There is contradictory information on the structural organization and catalytic properties of DNase I-like

proteins (especially DNase X and DNAS1L2). The molecular masses of these enzymes vary within 33-35 kD. Cleavage of the signal protein, which was found in all members of the DNase I family, was reliably demonstrated only for DNase γ [27, 89]. DNase γ and DNAS1L2 lack potential sites for N-glycosylation, whereas in DNase X this site is at Asn243. The presence of a C-terminal region of transmembrane domain is a structural feature of DNase X [86]. Polypeptide chain of DNase γ contains two nuclear localization signals (NLS), and so some authors suggest that under some conditions (e.g., during apoptosis) it may be translocated into the nucleus [27, 92].

Experiments with three purified recombinant DNases revealed that metal ion dependence and pH optimum of hydrolysis of double-strand DNA basically coincided with those found for DNase I [27]. DNAS1L2 is the only exception: this enzyme exhibits maximal activity at pH 5.6. Studies by Biunno's group demonstrated that DNase X share similarity with DNase II in metal ion dependence and pH optimum [88, 91]. Sensitivity to inhibition by aurintricarboxylic acid (ATA) is another parameter underlying the differences between DNase I-like enzymes. DNase X is less sensitive to ATA (IC_{50} = 38 μM) than the two other DNase I-like enzymes (IC_{50} = 0.4 μM) [27]. In contrast to DNase I, these nucleases are completely insensitive to inhibition by actin [27, 87, 90]. The biological role of DNase I-like nucleases remains to be clarified. These enzymes are now investigated for possible involvement (mainly DNase γ) in chromatin DNA cleavage during apoptosis [27, 92, 93].

DNase II

Human DNase II is a member of the mammalian DNase II family. Enzymes of this family are characterized by acidic pH optimum, lack of activator requirement, and formation of DNA reaction products with phosphate group at the 3′-end (EC 3.1.22.1) [6, 10]. Amino acid sequences of the DNase II family do not share significant homology with other proteins, whereas inside this family a high level of conservative residues exists. Identity of amino acids and their conservativeness are about 70% and >80%, respectively [94-96].

The gene encoding acidic DNase (*DNASE2*) consists of six exons separated by five introns; it is localized on the 19th chromosome in the p13.2 region [96, 97]. Only two allele variants of *DNASE2* are known to date [99]; this is attributed to substitution of guanine to adenine in a promoter region and such substitution results in fivefold reduction of expression [100].

The template transcript of DNase II has been found in almost all human tissues. This was confirmed by ELISA and assay of DNase activity in tissue extracts and lysates of cell lines [95, 98, 101-103]. In cells acidic DNase is preferentially localized in lysosomes, but DNase activity is also detected in secretory and other body fluids: saliva, blood, urine, and testicular liquid. Among 20 human cell lines the highest and the lowest DNase activity was found in epithelial and hematopoietic cells [103]. Blood and human urine are characterized by low content of DNase II; in urine its content is 30 times less than that of DNase I [102, 104, 105].

DNase II is a polypeptide consisting of 360 amino acids; the N-end of the enzyme contains a hydrophobic signal peptide, but cleavage of this peptide during post-translational modification has not been well documented [94, 103]. Data on DNase II processing in lysosomes (see [98, 106, 107]) are due to cleavage during enzyme isolation [103]. The amino acid sequence of DNase II contains seven Cys, six of them being highly conservative in all mammals, and formation of at least one S–S bond has been demonstrated [103]. The polypeptide chain contains four potential N-glycosylation sites (N86, N212, N266, and N290), which are essential for nuclease maturation and manifestation of catalytic activity [98, 106]. Cell cultivation in the presence of N-glycosylation inhibitors results in appearance of a lowly active form of this enzyme and reduction in molecular mass of the DNase from 45 to 37 kD during analysis in the Laemmli system [103]. Calculated molecular masses and isoelectric point for DNase II (without signal peptide) are 38 kD and pH 9.0, respectively [94]. Analysis of urinary acidic DNase by isoelectric focusing revealed several bands within the pH range 5-7 [102, 104]. Shift in p*I* value for the enzyme molecule into the acidic region may be attributed to the presence of sialic acid in a carbohydrate component, and possibly, mannose-6-phosphate; this is typical for lysosomal enzymes.

DNase II is a typical acidic hydrolase with pH optimum of 4.8-5.2; at pH 7 its catalytic activity is at least 100 times lower than at the pH optimum [94, 102]. Limited information is available on the structural organization of this enzyme and its mechanism of DNA cleavage. Site-directed mutagenesis revealed the presence of His295 in the active site of DNase II and the key role of this residue in manifestation of catalytic activity of this enzyme [103]. His295 is surrounded by highly conservative amino acid residues, which form the motifs S-T-E-D-H-S-K-W and A-T-E-D-H-S-K-W in humans and pigs, respectively [94]. Fe^{3+} and Cu^{2+} strongly inhibit the reaction rate, whereas magnesium, manganese, calcium, and zinc salts reduce the reaction rate only by 3-5-times [104, 108]. NaCl also influences the activity of DNase II; its total inhibition is observed at 0.4 M NaCl [102].

DNase II is a nonspecific endodeoxyribonuclease; this enzyme can hydrolyze phosphodiester bond between any nucleotides except the four terminal nucleotides at the 3′-end [108]. Under optimal reaction conditions (50 mM NaAc, pH 5.0, 1 mM EDTA), acidic DNase II is 5-10 times more active in hydrolysis of native double strand

DNA than in hydrolysis of denatured DNA; it cannot hydrolyze RNA [104]. Analysis of the catalytic mechanism of DNase II action on supercoiled DNA revealed that this enzyme preferentially introduces single strand breaks in both DNA strands [94, 108]. Stomach mucosa acidic DNase is probably the only exception: this enzyme shares similarity with pig spleen DNase in the ratio of single and double strand breaks [109]. DNase II is rather resistant to heating to 60°C; its treatment at 65°C for 10 min caused only two-fold reduction of catalytic activity and only heating to 75°C caused total inactivation [102, 104]. Analysis of DNase II activity in gel with polymerized DNA substrate (followed by separation in the Laemmli system) revealed that temperature treatment in the presence of SDS and a reducing agent caused irreversible denaturation [108].

DNase II plays metabolic and protecting roles in the human body; this is supported by lysosomal localization of this enzyme and acidic pH optimum. It is possible that this enzyme is involved in utilization of nucleosome DNA of apoptotic cells subjected to phagocytosis by macrophages [110, 111]. DNase II cannot be involved in replication, repair, and recombination of DNA because the reaction products carry 3′-terminal phosphate. Now certain evidence exists that DNase II may participate in cleavage of chromatin DNA during apoptosis, which is preceded by acidification of the cellular medium [112-114].

Recently a new gene encoding a protein consisting of 358 amino acid residues has been discovered; this gene has distinct chromosomal localization (at 1p22.3), and its protein product shares 37% of identity and 56% of conservativeness with DNase II [115, 116]. This enzymes has been denominated as DNase IIβ (DLAD) because its major catalytic characteristics of DNA hydrolysis are close to those of acidic DNase II (DNase IIα) [117]. In humans, the gene expression of DNase IIβ has been detected in salivary glands and to a lesser extent in lungs and prostate [115]. DNase IIβ contains an N-terminal signal peptide, nine cysteine residues, and four potential sites for N-glycosylation [103]. Certain evidence exists that DNase IIβ is present in salivary gland secretion because specific antibodies against DNase IIα suppress only half of the nuclease activity with acidic pH optimum [115].

PHOSPHODIESTERASE I

In humans and other mammals there is a whole family of phosphodiesterase I (EC 3.1.4.1, PDE I). Besides well-known PC-1 protein (PD-1γ), this family includes two recently discovered enzymes exhibiting similar catalytic properties [7]. Members of this family have alkaline pH optimum; they are also characterized by dependence of catalytic activity on bivalent metal ions (Me^{2+}). Originally, PC-1 (plasma cell protein) was found as a cell differentiation antigen during lymphoid cell transformation into antibody-producing cells [118]. In the beginning of the 1970s studies of PDE I activity in human organs and body fluids began [119-121], but only in 1991 identity of PC-1 and alkaline phosphodiesterase was recognized [122]. The gene encoding PC-1 is localized on the sixth chromosome within the q22-23 region [123, 124]. Now a new mutation in the coding region, which causes the substitution K121Q, is recognized, but its effect on enzyme structure and catalytic activity remains to be clarified [125, 126].

PC-1 has limited distribution in human organs and tissues. Template transcript of the phosphodiesterase I gene (*PDNP1*) was found in pancreas, uterus, heart, liver, and testicles; lower expression level was also detected in skeletal muscles, thymus, and prostate [127]. Several studies have revealed that chondrocytes and osteoblasts are characterized by relatively low level of basal expression of PC-1, but extracellular stimuli may significantly influence the expression level [128, 129]. Phosphodiesterase I activity has been found in the following body fluids: blood, urine, cerebrospinal liquid, testicular liquid, and milk [119, 130, 131]. In blood of healthy subjects, ELISA-analysis revealed the level of this enzyme of 36 ng/ml [132].

PDE I exists in two forms: the transmembrane homodimer (a type II ectoenzyme) and secretory monomer; their catalytic properties are almost indistinguishable [7, 133]. The membrane form of PC-1 contains at least one disulfide bond and so the molecular mass determined in the Laemmli electrophoretic system decreased from 230 to 130 kD after treatment with a reducing agent (Fig. 3) [7, 134]. The polypeptide chain consists of 925 amino acid residues; 76 amino acid

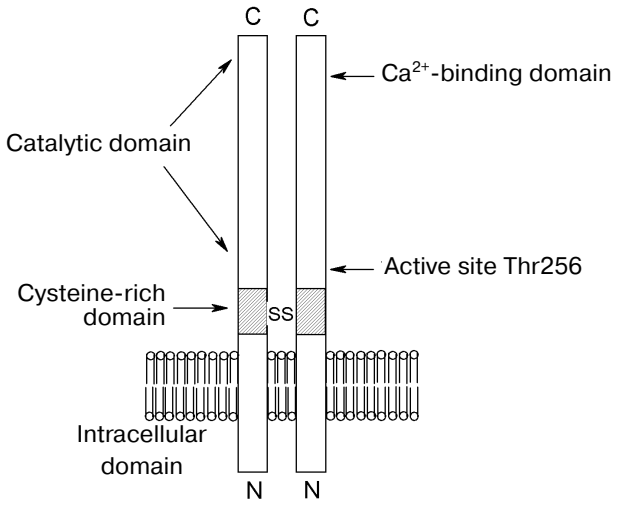

Fig. 3. Structure of PC-1 human membrane form [7].

residues form the cytoplasmic domain, 21 amino acid residues form the transmembrane domain, whereas the remaining 828 residues form the extracellular part of this enzyme. This region, including residues 97-925, contains an extended cysteine-rich site, several potential N-glycosylation sites, active site (containing Thr256), and also a calcium binding domain [7]. The secretory PC-1 form is originated during proteolytic cleavage of the N-terminal region of the protein molecule, which apparently occurs inside cells [134]. The enzyme looses a major fragment enriched with cysteines; it cannot form interchain disulfide bonds and consequently it exists in monomeric form. The molecular mass of the secretory PDE I is about 120 kD [133, 134].

The high glycosylation level of PC-1 results in overestimation of its molecular mass by 15-20 kD in the Laemmli system [134]. The major proportion of carbohydrate residues is of a complex type that is characterized by the presence of galactose, fucose, and sialic acid [134, 135]. Different content of sialic acid may account for significant polymorphism of blood alkaline phosphodiesterase by isoelectric point [121, 136, 137]. Ion-exchange chromatography or electrophoresis under native conditions may separate initial PDE I preparation into several isoenzymes differing in preference to P_i, butanol extraction, stability, and other parameters [138, 139]. Extracel-lular domains of human and mouse PC-1 share 80% identity in amino acid sequence, whereas the cytoplasmic domain is characterized by greater differences [134]. The active site contains the sequence T-F-P-N-H-Y, which is highly conservative in bovine, mouse, and human enzyme; this is consistent with previous data on close relations between human and other mammalian PDEs.

PDE I belongs to a class of nonspecific endonucleases: this enzyme cleaves a nucleoside 5′-phosphate at the 3′-end of DNA and RNA; it shows preference for single chain or denatured substrates [130, 134, 140, 141]. Cyclic mononucleotides, alkyl or aryl esters of nucleoside-5′-phosphates, and also phenylphosphonate esters are substrates for PDE I [130, 137, 142]. 4-Nitrophenyl-5′-phosphothymidine was used as substrate for determination of blood PDE I activity (K_m = 0.2 mM) [120, 143]. The enzyme does not exhibit monoesterase activity, and it cannot cleave the phosphotriester bond and also 4-nitrophenyl-3′-phosphothymidine, the standard substrate for PDE II [137, 140]. PC-1 is also a nucleotide pyrophosphatase (EC 3.6.1.9); it also exhibits pyrophosphohydrolase with respect to NAD, FAD, UDP-galactose, Ap_4A, ATP, and ADP [130, 138, 141]. PDE I is responsible for utilization of single strand DNA fragments in human blood; the half life time of deoxyribooligonucleotide is about 15 min, and hydrolysis involves only the 3′-end [140, 144]. The K_m value for pTAGCACCATGGTTTC is 50 μM and the rate of deoxyribooligonucleotide cleavage depends on the combination of the two last links (difference in the catalytic rate may reach two orders of magnitude between polar values [140, 145]).

PC-1 does not exhibit actual kinase activity as has been reported earlier [146], and radioactive enzyme labeling by [α-^{32}P]ATP may be attributed to intermediate covalent adduct (threonine-adenylate) formed during the reaction [7, 147, 148]. There are contradicting viewpoints on results of PC-1 labeling by [γ-^{32}P]ATP. Some authors believe in formation of a covalent bond between this enzyme and the nucleoside triphosphate [149]. Others suggest that this labeling may be attributed to terminal phosphate group transfer from ATP onto Thr located in the active site of PDE I [150]. Substitution of active site Thr for Ser, Ala, or Tyr results in a decrease in catalytic activity and ability of the mutant enzyme for autophosphorylation [149].

The pH optimum for PC-1 is at alkaline pH (pH 9-10 with 4-nitrophenyl-5′-phosphothymidine) and the precise value depends on substrate concentration [119, 120]. Bivalent ions Mg^{2+}, Ca^{2+}, and Zn^{2+} are usually used as cofactors of PC-1, but their relative activating potency has not been investigated [130, 136, 141]. Besides the active site, alkaline PDE also contains a special "EF hand" binding site for calcium ions, which significantly stabilizes the protein structure [151]. Metal ion chelation by EDTA totally inhibits the catalytic activity and reduces enzyme stability for proteolytic and thermal treatments [130, 151]. Protein inhibitors of phosphodiesterase/pyrophosphatase activity of PC-1 have not been discovered. The enzyme is sensitive to reducing agents [130, 141], whereas contradicting data have been reported on the effect of heparin on its activity [133, 140].

The biological functions of PC-1 remain poorly understood [7]. The membrane form of PDE I is one of the key ectoenzymes (together with 5′-nucleotidase and alkaline phosphatase) responsible for further degradation of nucleic acid fragments to nucleosides, which are then transported into cells. Hydrolyzing foreign DNAs and RNAs, this enzyme also plays a protecting role. PC-1 may influence extracellular concentrations of ATP and adenosine and, therefore, regulate the level of purinergic agonists and, consequently, cell response to these signal molecules. PDE I and alkaline phosphatase are the key enzymes regulating the concentration ratio of PP_i and P_i, the latter determining mineralization of cartilaginous and bone tissues [128, 152, 153]. Many attempts have been undertaken to find interrelationships between blood alkaline phosphodiesterase activity, its isoenzyme spectrum, and some diseases (mostly hepatic diseases) in humans [120, 121, 131, 143, 154, 155]. At the present time, the effect of the K121O mutation on the development of such diseases as myocardial infarction, liver insufficiency, etc. is actively investigated [126, 156-158]. During the last decade, many reports have been published on the mechanism by which PC-1 influences pathogenesis of insulin-resistance in patients with diabetes mellitus type 2 [159-162].

Properties of two other members of the PDE family are less studied; moreover, their oligonucleotide hydrolyzing activity remains to be investigated. The *PDNP3* gene, encoding a polypeptide chain of 875 amino acid residues for phosphodiesterase PD-1β, is located on the sixth chromosome within the q22 region; it is mainly expressed in prostate and uterus [7, 127]. The *PDNP2* gene located at the 8q24.1 locus is responsible for synthesis of two polypeptides, PD-1α and autotaxin, representing products of alternative splicing [163]. This gene is expressed in brain, small intestine, lungs, kidneys, placenta, and ovaries [7, 163, 164]. Autotaxin shares 45% identity with PC-1; it consists of 915 residues (this is 52 residues longer than that of PD-1α [163, 165]). Autotaxin is a N-glycoprotein of 125 kD with isoelectric point in the weakly alkaline region [166, 167]. At picomolar concentrations, it stimulates motility of tumor cells, and this activity is determined by the catalytic site of this enzyme (containing Thr210) [168]. Interestingly, autotaxin may cleave NTP at both positions of the β-phosphate [169]. Metal ion dependence of autotaxin phosphodiesterase activity coincides with that of PC-1 [170].

DNase DFF40

In 1997, an intensive search for nucleases involved in apoptotic cleavage of nuclear DNA culminated in the discovery of DNase DFF40 (DNA fragmentation factor) [171]. Other alternative names of this enzyme include CAD or CPAN (caspase activated nuclease). Expression of DFF40 mRNA was found in a limited number of organs such as pancreas, spleen, prostate, and ovaries [172]. DFF40 consists of a single polypeptide chain of 40 kD; its isoelectric point is at pH 8.7 [172, 173]. Formation of active form of this enzyme requires the presence of another protein known as DFF45; it acts as a chaperone during synthesis of DFF40. DFF45 is a specific inhibitor of DNA-hydrolyzing activity of DFF40 [174]. Inhibition occurs due to interaction of the catalytic domain of DFF40 with the D2-site of the DFF45 molecule followed by active site shielding [175]. The interaction of N-terminal domains sharing significant homology is the key event in DFF40/DFF45 complex formation, which buries a hydrophobic region inside the protein globule. During apoptosis, induction the activated caspase 3 cleaves DFF45 at two sites and the heterodimer DFF complex decomposes [171, 176]. Released DFF40 undergoes further oligomerization followed by formation of a large functional complex exhibiting high specific activity in DNA hydrolysis [173, 177]. The catalytic site of DFF40 including several conservative histidines is located at the C-end [178-180].

In the presence of Mg^{2+} ions (physiological pH and ionic strength values), DFF40 cleaves DNA with formation of double strand breaks [181]. Mn^{2+} and Ca^{2+} weakly activate the nuclease activity and Zn^{2+} and Cu^{2+} cause potent inhibition. Breaks in both DNA strands are either positioned opposite each other or shifted by one nucleotide; the latter results in formation of overhanging 5′-ends [182]. Hydrolysis of the phosphodiester bond yields 5′-phosphates. DFF40 activity depends on the ionic strength of the solution [181]: at optimal KCl concentration (50-125 mM), the rate of hydrolysis is 100 times higher than at 0 or 200 mM KCl. At high ionic strength, values the enzyme preferentially introduces single strand breaks (changes in oligomeric structure of the protein are not observed).

Under optimal conditions for hydrolysis, DFF40 does not cleave RNA and single strand DNA, and it also does not exhibit exonuclease activity [182]. Chromatin DNA cleavage sites correspond to internucleosome sites and cleavage results in formation of the apoptotic pattern of a DNA fragmentation ladder. Although DFF40 is a nonspecific endo-DNase it is more selective towards DNA sequence than DNase I and micrococcal nuclease. Sites for hydrolysis are characterized by double symmetry axis with respect to purine and pyrimidine content. Hydrolysis of DNA purified from chromatic proteins, histone H1, topoisomerase II, and other nuclear proteins significantly stimulate the nuclease activity [177, 182]. This enzyme is thermally unstable and is rapidly inactivated at 42°C [181].

ENDONUCLEASE G

Endonuclease G is a highly conservative protein of the mammalian family; this fact underlines almost total similarity of physicochemical and catalytic properties of this nuclease from various species [183, 184]. This enzyme is widely distributed in various human organs and tissue, where it is located in nuclear and mitochondrial fractions [185, 186]. The latest studies have revealed that in mitochondria endonuclease G is located in the intermembrane space rather than in the matrix [187]. The gene encoding this nuclease is located in the region 9q34.1 [188]. Native endonuclease G exists as a homodimer with molecular mass of subunits of 29 kD [189]. A leader sequence at the N-end of this enzyme representing a signal for its subcellular localization is removed during maturation of the protein. The enzyme is activated by Mg^{2+} and Mn^{2+} but not Ca^{2+} [189, 190]. Increase in ionic strength up to physiological values causes significant reduction of the reaction rate (by 16 times). The enzyme has two pH optima for hydrolysis of double strand DNA substrate, at pH 7.0 and 9.0. The latter value may be attributed to DNA melting and appearance of single strand structures, for which endonuclease G shows pronounced preference.

At high enzyme concentration and low ionic strength endonuclease G nonspecifically hydrolyzes sin-

gle strand and double strand DNA and RNA molecules and their hybrids with formation of acid-soluble products [185, 187, 189]. The location of single-strand breaks is not random in double strand DNA, and their accumulation results in formation of double strand breaks and 5′-phosphates. At low concentration, endonuclease G exhibits pronounced specificity towards guanine-rich sequences, which tend to form non-canonic triplex and quadruplex structures. The enzyme action on R-loop (the characteristic structure formed during initiation of replication) results in a double-strand break in DNA, whereas the RNA molecule remains intact [187]. The presence of phosphatidylcholine and phosphatidylethanolamine, the main phospholipid components of the inner mitochondrial membrane, causes 5-10-fold increase in DNA-hydrolyzing activity of this enzyme [191]. However, in the case of bovine endonuclease G it was demonstrated that high phospholipid concentrations (20-40 mM) significantly inhibited hydrolysis of nonspecific sequences [192].

The biological functions of endonuclease G remain unclear. Involvement of this enzyme in recombination and replication of mitochondrial DNA is now questioned [187, 193]. Certain evidence has recently appeared that endonuclease G participates in apoptotic cleavage of nuclear DNA [194].

LACTOFERRIN

Lactoferrin, one of biologically important components of mammalian milk, belongs to the family of transferrins. These glycoproteins transfer Fe^{3+} ions. Lactoferrin is a single polypeptide chain of 673 residues; its molecular mass varies within 76-80 kD. The lactoferrin molecule consists of two homologous domains, denominated as N- and C-lobes [195]. Each lobe contains one Fe^{3+}-binding site and one site for potential glycosylation. In human milk, lactoferrin saturation with iron does not exceed 10% and most of the properties of this protein are iron-independent [196].

In humans, lactoferrin can also be detected in epithelial secretions, barrier fluids (tears, saliva, etc.), blood, and urine. Lactoferrin is a unique polyfunctional protein. It can influence processes of cell proliferation and differentiation and regulate granulopoiesis. In some cells, lactoferrin stimulates DNA synthesis, and it inhibits prostaglandin biosynthesis in human milk macrophages. It also activates nonspecific immune response by stimulating phagocytosis and complement. Lactoferrin can interact with DNA, RNA, proteins, polysaccharides, and heparin-type polyanions and some lactoferrin functions are realized only in complexes with these ligands [197, 198]. Recently, it was demonstrated that lactoferrin can exhibit ribonuclease activity [199] and act as a transcription factor. These properties have been reviewed earlier [196].

Recently, it was found that lactoferrin possesses five different catalytic activities: RNase, DNase, phosphatase, ATPase, and amylase; it is also a major nuclease in human milk [11]. Chromatographic separation of homogenous preparation of human milk lactoferrin on Sepharose Blue yielded five fractions enriched in different catalytic activities. Among these partially separated forms of lactoferrin, two of them exhibited DNase activity. DNase activity of lactoferrin was maximal at neutral pH and physiological ionic strength. DNase activity assayed with oligonucleotide and double strand DNA was stimulated by Ca^{2+}, Cu^{2+}, Zn^{2+} (3-5-fold), and Mn^{2+} or Mg^{2+} (8-9-fold) ions; ATP, dATP, and NAD also stimulated the reaction rate by 1.5-2.5-fold. Lactoferrin was 30-200 times more effective in cleavage of plasmid double strand DNA (k_{cat} = 2-9 min^{-1}), than that of oligonucleotide. Using a set of various homooligonucleotides (K_m = 3.0-5.0 μM; k_{cat} = 0.026-0.029 min^{-1}), it was demonstrated that DNase activity of lactoferrin is sequence-nonspecific.

Use of various methods such as limited trypsinolysis, affinity modification with oligonucleotide derivatives, and in gel activity revealed that the DNase-hydrolyzing site is localized in the N-terminal lobe of lactoferrin [11]. This part of lactoferrin also contains two DNA-binding sites (exhibited different affinity to DNA) and a polyanion-binding site [197, 200]. Interestingly, lactoferrin fraction exhibiting DNase was characterized by more pronounced cytotoxic effect on mouse fibroblast tumor cell line L929 and human promyelocyte HL-60 cells; treatment of these cells with 100 nM lactoferrin caused the development of typical pattern of cleavage of nuclear DNA [11].

IMMUNOGLOBULINS EXHIBITING DNase ACTIVITY

The possibility in principal of production of catalytically active antibodies or abzymes was proposed by Pauling in 1948, who had noted common features between recognition mechanisms underlying antigen–antibody interaction and the interaction of enzyme with transition state of substrate [201]. In 1969, Jencks suggested the possibility of production of abzymes as antibodies against stable analogs of transition states [202]. In 1986, two groups (Tramontano et al. [203] and Pollack et al. [204]) provided direct experimental evidence supporting this suggestion. During the last thirty years, this approach has been successfully employed for directed synthesis of abzymes catalyzing more than 100 various chemical reactions (see for review [3, 205, 206]).

Natural catalytically active antibodies have been found in blood of patients with bronchial asthma [207]. Later abzymes with DNase activity were also detected in blood of patients with systemic lupus erythematosus

[208]. Production of abzymes exhibiting various protein, DNA, RNA, and polysaccharide-hydrolyzing activities was found in patients with autoimmune diseases and some viral diseases (see reviews [3-5]). Now DNA-hydrolyzing IgG and/or IgM have been recognized in blood of patients with such autoimmune diseases as multiple sclerosis [209], thyroiditis [210], rheumatoid arthritis, and systemic scleroderma [211], and also viral hepatitis [212] and AIDS [213]. Moreover, DNA-hydrolyzing abzymes have been found in blood of healthy pregnant and lactating women [214-217]. Using antibodies isolated from blood of patients with systemic lupus erythematosus, multiple sclerosis, and also from milk of postpartum women, it was found that DNase activity was attributed to both intact IgG molecules and their Fab-fragment and isolated light chains [209, 218, 219]. In most cases, abzyme active sites are located on light chains, but sometimes both light and heavy chains may be involved in organization of the catalytic site. For example, the DNA-binding site of human milk sIgA is preferentially located on heavy chains, whereas the catalytic site is on light chains [214].

Certain evidence now exists that production of DNA-hydrolyzing abzymes involves two paths [3-5]. On one hand, such antibodies are antiidiotypic to active sites of nucleases circulating in blood. On the other hand, DNA-hydrolyzing antibodies may represent antibodies produced against DNA or DNA–protein complexes, which are released in large quantities into the circulation in autoimmune diseases. The predominance of each path depends on type of disease, stage of its development, and features of the immune system of the patient [3-5]. The level of relative activity of polyclonal abzymes significantly varies from patient to patient and their repertoire extends during progression of the disease and attack of autoimmune disease [3-5]. For example, polyclonal antibodies in patients with multiple sclerosis may contain a few types of DNase activity: various combinations of endo- and exonuclease activities, sensitive or insensitive to activation by metal ions [220]. DNA-hydrolyzing abzymes may be partially separated into subfractions during affinity chromatography on DNA affinity sorbents [209]. Abzyme fractions with various affinities to substrates usually demonstrate marked differences in all kinetic and thermodynamic characteristics. Moreover, monoclonal antibodies against DNA are 50-100 times more active in hydrolysis of RNA than DNA [221]. Thus, it is possible that in contrast to ordinary enzymes, natural abzymes may simultaneously act as DNases and RNases with a universal hydrolytic center.

In patients with studied pathological autoimmune states, the relative activity of antibodies in DNA cleavage varies over a wide range. However, the ratio of species number of antibodies characterized by high, moderate, and low activity depends on type of disease and the following trends have been found for increase in abzyme activity: hepatitis ≤ AIDS ≤ autoimmune thyroiditis ≤ polyarthritis < systemic lupus erythematosus ≤ multiple sclerosis [3-5]. Analysis of relative DNase activity of antibodies may serve as an additional criterion during diagnostics of autoimmune diseases even at early stages of their development [222]. Detailed properties of abzyme functioning (including DNA-hydrolyzing antibodies) have been considered earlier [3-5].

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