

Knock down of cytosolic phospholipase A2: an antisense oligonucleotide having a nuclear localization binds a C-terminal motif of glyceraldehyde-3-phosphate dehydrogenase

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Abstract

We have previously shown that an antisense, effective in the knock down of cytosolic phospholipase A2 (cPLA2), localizes mainly in the nucleus of human endothelial cells and monocytes and that glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is involved in its nuclear localization. In this study, we clarify how GAPDH participates in the nuclear localization of this antisense oligodeoxynucleotide (ODN) directed against cPLA2 mRNA. A central TAAAT motif providing specificity and high affinity binding was assumed to interact with the enzyme Rossmann fold region on the basis of competition to this site by NAD⁺. To assess whether the TAAAT motif interacts directly with the enzyme Rossmann fold region, we evaluated the binding to GAPDH of different oligonucleotides and the effect of competitors such as NAD⁺, NADH, mononucleotides, DNA, polyribonucleic acids and polyanions. We found that the dissociation constant for TAAAT containing oligonucleotides was three - to fivefold higher with respect to oligo not containing this motif. By covalently linking ³²P-labeled cPLA2p(N)₁₆ to GAPDH and after executing hydrolysis with hydroxylamine, the labeling was exclusively found in the C-terminal domain (aa 286–334). These results indicate that the antisense oligonucleotide interacts with a site not having a defined function but which can be negatively allosterically regulated when NAD⁺ or polynucleotides are bound to Rossmann fold.

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1. Introduction

In this study we used an anti-cytosolic phospholipase A2 (cPLA2) oligodeoxynucleotide (ODN) able to efficiently knock down the protein level and the enzymatic activity of the enzyme, as previously evaluated [1]. An interesting characteristic of this antisense is that, when placed in the culture medium without transfecting agents, it is rapidly taken up by human primary cells and accumulates in the nucleus. In a previous study we have shown that this antisense ODN interacted with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and that a TAAAT motif represents a major binding sequence to GAPDH. Moreover, the binding of this ODN to GAPDH was responsible for its rapid nuclear accumulation [2,3].

For over three decades, GAPDH was studied for its pivotal role in glycolysis and was intensively investigated as a model for examining basic mechanisms of enzyme functions as well as the relationship between amino acid sequence and protein structure [4,5]. Amino acids critical for NAD⁺ and glyceraldehyde-3-phosphate (G3P) binding, their spatial arrangement and interactions during functioning of the enzyme were examined [6,7]. Later, intensive biochemical studies demonstrated the importance of GAPDH for membrane transport, membrane fusion, microtubule assembly, nuclear RNA export, translational control of gene expression, DNA replication and DNA repair [6,8–12]. Moreover, evidence proving a role of GAPDH in apoptosis, prostate cancer development and CAG triplet disorders were obtained [13–18]. This functional redundancy implies the existence of specific interactions of the protein with different cellular ligands, which may affect GAPDH structure, alter its functions and induce pleiotropic changes in mammalian cells.

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The interactions of GAPDH with RNA and DNA have been described earlier [19,20]. It was shown that GAPDH binds tRNA, LTR of different viral RNAs, AU-rich elements of mRNA and participates in nuclear RNA transport. The AUUUA-specific RNA binding activity of GAPDH is inhibited by NAD^+ , NADH and ATP in a concentration-dependent manner, suggesting that RNA binding of GAPDH may involve the NAD^+ binding region (Rossmann fold) [2,21–27]. By searching for RNA-binding domain with GST-GAPDH fusion proteins generated by deletion mutagenesis, we have demonstrated that the N-terminal 43-amino-acid fragment of GAPDH corresponding to the first mononucleotide binding domain of NAD^+ is sufficient to confer RNA-binding [2].

We previously established that GAPDH is involved in the nuclear localisation of an antisense ODN directed against cPLA2 mRNA [2]. This ODN is efficiently taken up and accumulates in the nucleus of endothelial cells (HUVE), human monocytes and HeLa cells. Gel shift experiments and incubation of cells with oligonucleotide derivatives showed that the anti cPLA2 oligo binds a 37-kDa protein in nuclear extracts. TAAAT sequence was identified as the major binding motif for the nuclear protein in competition experiments with mutated ODNs. The 37-kDa protein was purified and identified after peptide sequencing as GAPDH. It was shown by confocal microscopy that GAPDH co-localises with anti cPLA2 ODN in the nucleus and commercial GAPDH efficiently binds the oligonucleotide [2,3]. As previously shown, NAD^+ negatively regulates the binding of cPLA2 ODN to GAPDH. However, it is not clear whether a competition for the same site or an allosteric effect was involved. The identification of a novel C-terminal site specific for the antisense favours an allosteric role for NAD^+ .

2. Materials and methods

2.1. Synthesis of oligonucleotides

Oligonucleotides were synthesised by using an ASM-102U DNA Synthesiser (BioSet, Russia) by the phosphoramidite method. ODNs were purified by using oligonucleotide purification cartridges (Perkin Elmer, USA) followed by electrophoresis on 20% polyacrylamide gel, containing 6 M urea, and electroelution. A 16-mer oligonucleotide (3' - CAGTAAATATCTAGGA-5') directed against the translation initiation site of cPLA2 mRNA was used as a specific binding oligonucleotide (cPLA2p(N)₁₆). Heterogeneous oligonucleotide pATAAGCAACAAGCCCT and homopolymer p(T)₁₆ were used for estimation of dissociation complexes of GAPDH with ODNs.

The 5'-end of oligonucleotides was radiolabeled with [³²P] using T4 polynucleotide kinase (Gibco, USA). The cetyltrimethylammonium salt of [³²P]ODN was 5'-labeled with the 4-[(N-2-chloroethyl-N-methyl)amino]benzylamine

alkylating group after activation of its 5'-end phosphate with triphenylphosphine and dipyrindyl disulfide in a non-aqueous solution [28], thus producing [³²P]CIRpODN. Active chlorine was determined in the alkylating group by reaction with 0.5 M sodium thiosulfate for 10 h at room temperature. The yield of alkylating derivatives was higher than 90%, as indicated by electrophoresis of the reaction mixture on 20% PAGE containing urea, and specific radioactivity was around 50 Ci/mmol.

2.2. Determination of the dissociation constant

For the estimation of the dissociation constant of ODN–protein complexes, commercial enzyme aliquots were incubated with 0.01–2.5 μM [³²P]CIRpODNs in 50 mM Tris–HCl, pH 7.9, 1 mM EDTA, 1 mM PMSF and equal amounts of proteins were fractionated by 10–20% SDS-PAGE. Fragments of the gel containing modified proteins were cut off, and their radioactivity was detected by β -counting. The concentration of alkylating ODN derivatives was plotted as a function of the intensity of the bands obtained.

2.3. Competition experiments

Commercial GAPDH (1 μM), purified from human erythrocytes (Sigma, USA), was incubated with 1 μM [³²P]CIRcPLA2p(N)₁₆ for 1 h at 37 °, in the presence of fivefold molar excess of ATP, GTP, AMP, GMP, NAD^+ , NADH, G3P, cPLA2p(N)₁₆, or 15-fold weight excess of dsDNA, polyA, polyU, polyC, polyIC, heparin, dextran S. Modified proteins were separated by 10–20% SDS-PAGE and visualised by autoradiography of the gel. Fragments of the gel containing modified proteins were cut off and their radioactivity was detected by β -counting.

2.4. Effect of cPLA2p(N)₁₆ on dehydrogenase activity of GAPDH

The initial rate of NAD^+ reduction by GAPDH was determined at 7.5 and 15 μM NAD^+ concentrations without cPLA2p(N)₁₆ or in the presence of 7.5, 15 and 30 μM cPLA2p(N)₁₆. Forward reaction was carried out in 24 mM sodium pyrophosphate (pH 8.5) containing 4 mM cysteine, 17 mM disodium arsenate, 1.4 mM G3P. The reaction was started after addition of GAPDH (0.5 $\mu\text{g}/\text{ml}$, specific activity 50 U/mg) and performed at 25 °C. The reduction of NAD^+ was measured by using a spectrophotometer, during the first 2 min, as the increase in absorbance at 340 nm.

2.5. UV cross-linking assay

[³²P]-labeled cPLA2p(N)₁₆ (1 μM solution) was incubated with 1 μM GAPDH for 40 min at room temperature. Open polypropylene Eppendorf tubes, containing the reaction mixture, were exposed to UV generated by a standard Philips TUV 15-W lamp for 40 min on ice at the distance of 5 cm.

2.6. Hydrolysis of modified GAPDH

To hydrolyse asparagine–glycine bond, modified GAPDH was incubated for 12 h with 2% hydroxylamine hydrochloride, titrated to pH 9.0 with LiOH in 6 M guanidine chloride, dialysed, precipitated with acetone and dissolved in sample buffer. Obtained polypeptides were separated in 15–20% SDS-PAGE.

3. Results

3.1. Determination of the dissociation constant

The dissociation constant (K_d) for the binding GAPDH-oligonucleotides was evaluated using alkylating ODN derivatives as described [29,30]. For CPLA2pN16, a K_d value of 0.2 μM was calculated while for pATAAGCAACAAGCCCT

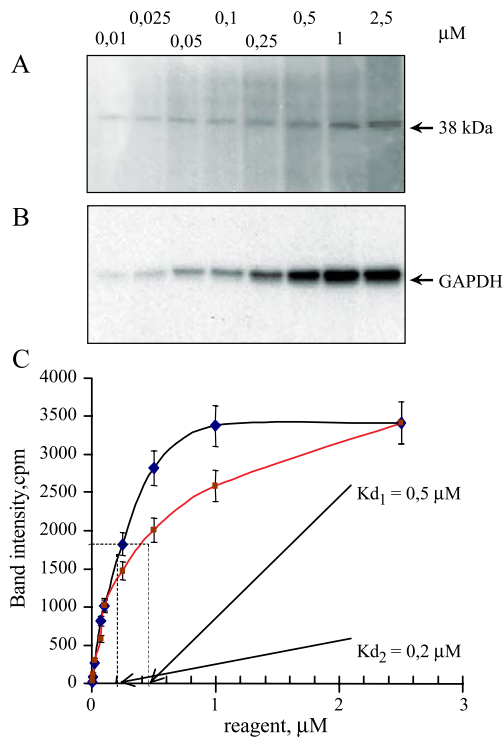


Fig. 1. Estimation of the dissociation constant (K_d) of ODN-GAPDH complexes. Nuclear extract of HeLa cells (panel A) or commercial purified GAPDH from human erythrocytes (panel B) was incubated with increasing concentrations of [^{32}P]CIRcPLA2p(N) $_{16}$ (0.01–2.5 μM) for 1 h at 37 °C. Modified proteins were separated by 10–20% SDS-PAGE and the 38-kDa band was visualised after exposure to X-ray film at -80 °C with intensifying screen. In panel C the concentration of the alkylating ODN derivative was plotted as a function of gel band intensity, which was quantified by using a β -counter, as described in Materials and methods. Each point represents the mean \pm S.E. of three values. The data fitted with a single binding site model and the dissociation constants (K_d) of ODN–protein complexes were determined from a double-reciprocal plot as previously described [28,46]. K_{d1} represents the dissociation constant of the nuclear GAPDH–ODN complexes, while K_{d2} represents the dissociation constant of the erythrocyte-purified GAPDH–ODN complexes.

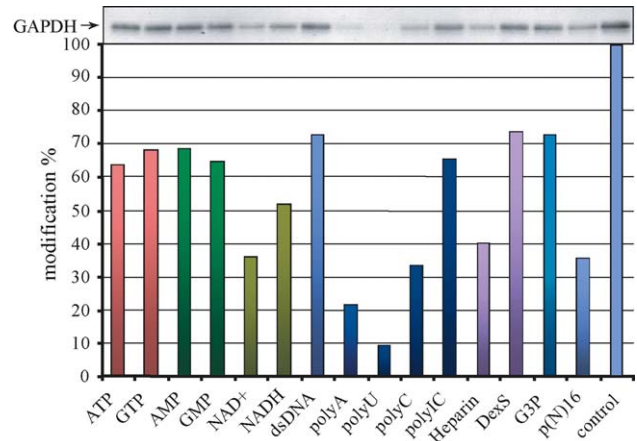


Fig. 2. Effects of competitors on the yield of the modification of GAPDH with [^{32}P]CIRcPLA2p(N) $_{16}$. GAPDH was incubated with 1 μM [^{32}P]CIRp(N) $_{16}$ 1 h at 37 °C in presence of 5 \times molar excess of ATP, GTP, AMP, GMP, NAD $^+$, NADH, G3P, p(N) $_{16}$, or 15-fold weight excess of dsDNA, polyA, polyU, polyC, polyIC, heparin, DexS. Modified proteins were separated by 10–20% SDS-PAGE and visualised by autoradiography of the gel. Fragments of the gel containing modified proteins were cut off, and their radioactivity was detected by β -counting. The experiment was repeated with essentially similar results.

and homopolymer p(T) $_{16}$ values ranging from 1 to 2 μM were found (Fig. 1). Thus, the binding GAPDH-oligonucleotides we detected had an higher affinity value with respect to other tested oligonucleotides. This high affinity value, comparable to that detected for other cellular ODN-binding proteins studied to date [9,30], could promote exchange of oligonucleotides between low affinity complexes of ODNs with cell surface proteins and GAPDH.

3.2. Competition experiments

Experiments were performed using [^{32}P]CIRcPLA2p(N) $_{16}$ and GAPDH purified from human erythrocytes as previously described [2] (Fig. 2). A strong competition was observed using polyU and polyA while NAD $^+$, polyIC and heparin partially competed. Dextran sulfate, ATP, AMP, GTP, GMP, G3P, NADH and dsDNA weakly inhibited the binding reaction between [^{32}P]CIRcPLA2p(N) $_{16}$ and GAPDH. It should be noted that incubation of the enzyme with [^{32}P]CIRcPLA2p(N) $_{16}$ in the presence of 50-fold molar excess of these compounds decreases affinity of the enzyme no more than 10% of presented values (data not shown). Decrease of affinity modification of GAPDH with [^{32}P]CIRcPLA2p(N) $_{16}$ in the presence of polyanions like heparin suggests the involvement of ionic interactions between GAPDH and oligonucleotide.

The high concentrations of NAD $^+$ required to antagonize [^{32}P]CIRcPLA2p(N) $_{16}$ binding to GAPDH previously detected and confirmed here, and the fact that at 10 μM concentration NAD $^+$ completely inhibits GAPDH binding to RNA [2] but not GAPDH-oligonucleotide binding, strongly suggest that the antisense used does not occupy the Rossmann fold of the enzyme.

We have demonstrated that the extent of modification of GAPDH with [32 P]ClRcPLA2p(N) $_{16}$ was fivefold higher in buffer for determination of dehydrogenase activity (24 mM sodium pyrophosphate, pH 8.5, containing 4 mM cysteine, 17 mM disodium arsenate) than in buffers generally used for determination of uracil DNA glycosylase activity of the enzyme, even if the dissociation constants of the complexes GAPDH–ODN coincide for both buffers. It was suggested that dissociation of tetrameric GAPDH to the monomeric form was responsible for the decrease of dehydrogenase activity and that arsenate prevented loss of enzyme activity [28]. Hence, oligonucleotides obviously prefer to interact with di/tetrameric form of the enzyme. A weak competition of the binding cPLA2p(N) $_{16}$ /GAPDH by G-3-P could be explained by conformational changes of the enzyme after substrate binding.

3.3. Effect of cPLA2p(N) $_{16}$ on dehydrogenase activity of GAPDH

The failure of G-3-P to affect the binding reaction suggests that also the enzyme active site is not involved in recognizing the antisense oligo. Therefore, the influence of cPLA2p(N) $_{16}$ on GAPDH dehydrogenase activity was investigated at 7.5, 15 and 30 μ M doses. As the dissociation constant of complex of GAPDH with cPLA2p(N) $_{16}$ is 0.2 μ M, all molecules of the enzyme should form complexes with oligonucleotide. Dickson linear plot obtained from experimental points of the competition of cPLA2p(N) $_{16}$ binding with GAPDH enzymatic activity demonstrates that a weak uncompetitive inhibition of dehydrogenase activity of GAPDH occurs (Fig. 3). These data suggest that the

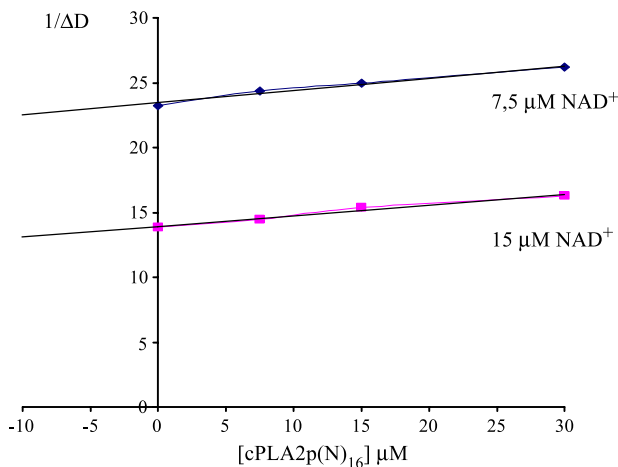


Fig. 3. Effects of p(N) $_{16}$ on enzymatic activity of GAPDH. Initial rate of NAD $^{+}$ reduction by GAPDH was determined at different NAD $^{+}$ concentrations (7.5 or 15 mM) in the presence of p(N) $_{16}$ oligonucleotide at different concentrations (0, 7.5, 15 and 30 mM). Reaction was started after addition of GAPDH as described under Materials and methods, and performed at 25 $^{\circ}$ C. Reduction of NAD $^{+}$ was measured during the first 2 min and was determined as the increased absorbance at 340 nm. Figure shows the Dickson linear plot of the experimental points obtained. The experiment was repeated with essentially similar results.

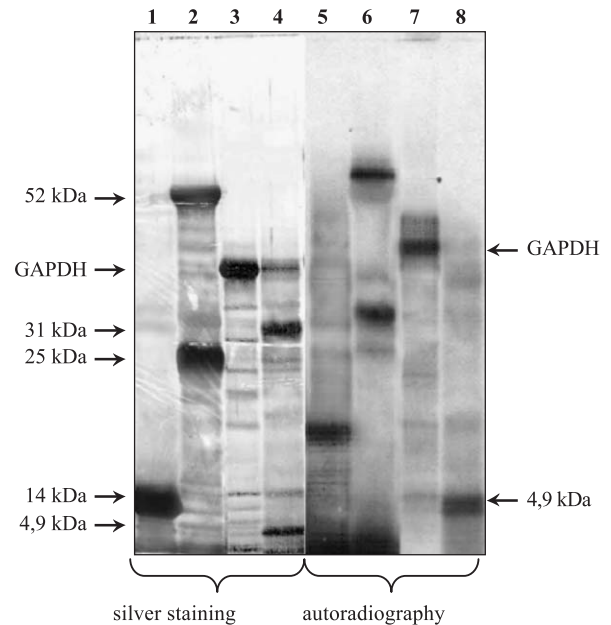


Fig. 4. Identification of oligonucleotide-binding region of GAPDH. Purified GAPDH from human erythrocytes, modified with [32 P]p(N) $_{16}$ by UV-irradiation, was treated with hydroxylamine. Products of hydrolysis of GAPDH were analyzed by 15–20% SDS-PAGE, followed with nitrocellulose blot staining with Silver (on the left) or followed by autoradiography (on the right). Lanes 1, 2, 5, 6 show molecular weight marker proteins, respectively, lysozyme (14 kDa) and IgG (52, 25 kDa), modified with [32 P]ClRc(N) $_{16}$. Lanes 3 and 7 show GAPDH, modified with [32 P]p(N) $_{16}$. Lanes 4 and 8 show the product of hydroxylamine hydrolysis of GAPDH, modified with [32 P]p(N) $_{16}$. The experiment was repeated with essentially similar results. Higher molecular weight very faint bands (lane 8) are likely artifacts representing nonspecific binding.

oligonucleotide binding site differs from sites responsible for enzymatic activity.

3.4. Detection of cPLA2p(N) $_{16}$ binding site

To approach this point, GAPDH was affinity-modified with [32 P]cPLA2p(N) $_{16}$ using 260-nm UV irradiation. As opposed to affinity modification with alkylating derivative of oligonucleotide, this kind of modification leads to the formation of covalent bonds in places of tight contacts between the oligonucleotide and the enzyme. Hydrolysis with hydroxylamine in the conditions described under Materials and methods leads to the formation of two polypeptides: an N-terminal one, having a molecular mass around 31 kDa, and a C-terminal one, having a molecular mass around 5 kDa. Only the polypeptide 286–334 was radiolabeled, demonstrating that the oligonucleotide binding site differs from Rossmann fold (Fig. 4).

4. Discussion

The 85-kDa cPLA2 is an enzyme central for the regulation of different cell functions, such as vascular cell prolif-

eration or inflammatory responses [31,32]. The pharmacological use of cPLA2 inhibitors has been proposed for restenosis treatment and other pathologies related to vascular injuries [32]. Unfortunately, no specific inhibitor of this enzyme is available and antisense strategy represents an interesting way to develop specific inhibitors. Encouraging results have been obtained by using ODNs to modulate cPLA2 expression and activity in several experimental models [1,32–36]. Nevertheless, the mechanism of action of these anti-inflammatory ODNs has not been investigated. The results reported in this study strongly support our previous findings about the role of GAPDH in the nuclear delivery of ODN possessing a TAAAT motif. It is likely that GAPDH functions as a shuttle for the ODN and that its nuclear entry is facilitated by the presence of a nuclear localization signal (aa 259–263) and of a signal located in the Rossmann fold dictating the binding to pre-mRNA molecules concentrated inside PML-like nuclear bodies. In this way, the antisense ODN is strategically located to react with complementary sequences as AUUUA, detected in cPLA2 pre-mRNA [31,37]. This may result either in DNA/RNA duplex formation causing degradation of pre-mRNA or in the failure of cPLA2 pre-mRNA to be spliced and exported to the cytosol. The interest in this class of antisense oligonucleotides, having a nuclear site of action, is connected to the fact that the block in pre-mRNA maturation may cause a more prolonged down-regulation of protein expression with respect to ODNs acting by a classical antisense mechanism.

The versatility of GAPDH in the binding of proteins is highlighted by the demonstrations regarding huntingtin and ataxin 1 [38,39]. Both proteins contain polyglutamine domains which are expanded in the protein pathological version. Burke et al. [38] and Koshy et al. [39] suggest that interaction with long polyglutamine tracts may disrupt GAPDH activity interfering with energy metabolism. We propose that binding of altered huntingtin and ataxin 1 to GAPDH Rossmann fold, possibly favoured by transglutaminase cross-linking [40], may impair normal functions of GAPDH at the nuclear level [2,6,37] or, if the binding occurs in the cytosol, may interfere with the shuttling role of GAPDH from cytosol to the nucleus. Both possibilities are consistent with findings revealing an alteration in nuclear GAPDH structure observed in Huntington's disease and other neurodegenerative disorders [41–43]. Moreover, Schultze et al. [44] disclosed that β -amyloid precursor protein also binds GAPDH at its C-terminal end. These observations, at the light of our findings, indicate that proteins and nucleic acids can compete for a common binding site in the GAPDH molecule (Fig. 5). The functional relevance of ataxin–GAPDH interaction is strengthened by the presence of both proteins in nuclear PML bodies [2,45]. GAPDH is a protein that behaves as a sequence-specific binding protein, and could be one of the unidentified carrier proteins involved in the nucleus–cytoplasm shuttling of antisense oligonucleotides. In this

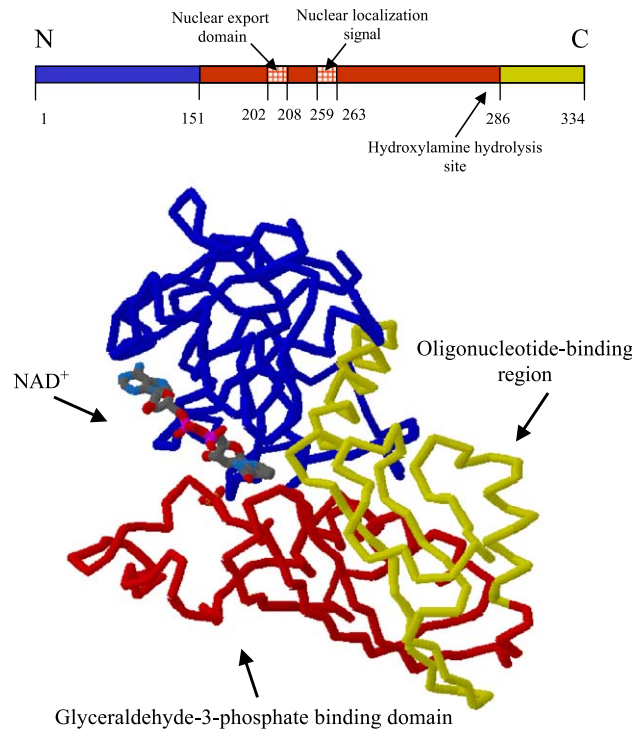


Fig. 5. Structural features of human GAPDH. The 2D (upper) and 3D (lower) structures of GAPDH are shown. Assignments of secondary and tertiary structures are consistent with findings reported in the cited literature. Structurally and functionally important regions are indicated. The NAD^+ binding domain is represented in blue, the glyceraldehyde-3-phosphate binding domain is represented in red, while the oligonucleotides binding domain is shown in light green.

study, we demonstrate that a specific region of GAPDH, different from the Rossmann fold and the active site of the protein, is involved in the binding of TAAAT containing ODNs. The presence of the TAAAT sequence appears to be a necessary, but not sufficient, condition for targeting an antisense into the nucleus. In fact, modification of this sequence resulted in ODNs which failed to localise in the nucleus and inserting TAAAT motif into an ODN, localizing in the cytosol, did not modify its localization (Tomasi et al. unpublished data). An issue we are facing is to try to understand whether TAAAT containing ODNs, able to interact with GAPDH, may be more effective than others in the inhibition of cPLA2 expression *in vivo*. If this is the case, these antisense ODNs would be very useful in the treatment of inflammatory diseases, even if it is necessary to underline that the interaction between ODN and GAPDH might be responsible for biological effects unrelated to cPLA2.

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