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The sweet side of RNA regulation: glyceraldehyde-3-phosphate dehydrogenase as a noncanonical RNA-binding protein

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Abstract

The glycolytic protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), has a vast array of extraglycolytic cellular functions, including interactions with nucleic acids. GAPDH has been implicated in the translocation of transfer RNA (tRNA), the regulation of cellular messenger RNA (mRNA) stability and translation, as well as the regulation of replication and gene expression of many single-stranded RNA viruses. A growing body of evidence supports GAPDH–RNA interactions serving as part of a larger coordination between intermediary metabolism and RNA biogenesis. Despite the established role of GAPDH in nucleic acid regulation, it is still unclear how and where GAPDH binds to its RNA targets, highlighted by the absence of any conserved RNA-binding sequences. This review will summarize our current understanding of GAPDH-mediated regulation of RNA function.

INTRODUCTION

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, E.C. 1.2.1.12) was initially characterized as a homotetrameric oxidoreductase playing a critical role in intermediary metabolism, wherein it catalyzes the sixth step of glycolysis by converting glyceraldehyde-3-phosphate (G3P) into 1,3-bispho-sphoglycerate. GAPDH is a multifunctional protein implicated in a variety of other cellular processes beyond metabolism, including membrane fusion,[1] cytoskeletal dynamics,[2,3] receptor-mediated cell signaling,[4] heme metabolism,[5,6] apoptosis,[7,8] DNA replication and repair,[9-11] and maintenance of genomic integrity.[12-14] In addition, GAPDH has a multiplicity of roles in dictating RNA localization, catalysis, translation, and replication (reviewed in Refs 15–22).

Mirroring this multitude of cellular functions, GAPDH has been shown to localize to various cell compartments. GAPDH is predominantly cytoplasmic, often associated with polysomes and cytoskeletal components, but is also found in the nucleus and in the mitochondria.[20] Its cytoplasmic distribution is due in part to an exportin 1-binding site (residues 259–271), which directs its nuclear export in the absence of stress.[23] Conversely, GAPDH responds to various stresses by associating to effector proteins and translocating to the nucleus, leading to apoptosis.[7,8,23-25] Other studies have shown that nuclear localization of GAPDH is

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regulated by a cell-cycle dependent mechanism, and that nuclear GAPDH may differ from cyto-plasmic GAPDH.[12] In addition, dimeric/non-native forms of nuclear GAPDH[12,26] have been observed, suggesting that GAPDH functions may be correlated with discrete structural determinants. The mechanisms by which GAPDH switches among these various functions and locations in the cell are unknown, but may depend on cell status, GAPDH posttranslational modifications, and its interaction partners.

An important extraglycolytic function of GAPDH is its binding to nucleic acids. GAPDH binding to single-stranded DNA was first reported in 1977 by Perucho et al.[27] Shortly after, GAPDH was found to bind to a wide range of DNAs and RNAs.[28–30] Since then, GAPDH has unambiguously emerged as a noncanonical RNA-binding protein (RBP) that binds a multitude of RNA scaffolds, including transfer RNA (tRNA), cellular RNAs, ribo-zymes, and viral RNAs. The diversity of GAPDH-RNA interactions is paralleled by the variety of their effects on RNA function.

In this review, we will survey the RNA-binding function of GAPDH, its possible involvement in the regulation of protein expression, the potential link between cellular energetics and RNA biogenesis, and the possible RNA-binding sites of GAPDH.

MESSENGER RNA-MEDIATED CONTROL OF GENE EXPRESSION

Dynamic control of gene expression levels is essential for cellular responses to internal and external stimuli.[31,32] Posttranscriptional control of eukaryotic gene expression comprises several levels of regulation including messenger RNA (mRNA) processing, export, turnover, and translation such that steady-state mRNA levels are not solely determined by the rate of mRNA synthesis. The fate of mRNA transcripts is determined in part by adenine–uridine rich *cis*-acting regulatory elements (AREs) located within the 3′ untranslated regions (3′ UTRs)[33] of cytokines, transcription factors, and proto-oncogenes.[34] AREs serve as binding sites for ARE-binding proteins (AUBPs), which are *trans*-acting factors in the regulation of ARE-dependent mRNA turnover and translation.[35-37] AUBP binding can decrease, as in the case of Tristetraprolin (TTP),[38] or enhance, as in the case of HuR[39,40] and Hsp70,[41] mRNA stability and may induce the formation of a complex of proteins at the ARE.[42,43] Emerging evidence suggests that some AUBPs, such as AUF-1,[44] can either stabilize or destabilize transcripts, depending on the specific mRNA bound.[45] In addition, as several AUBPs can target the same AREs, the enhancement or inhibition of mRNA stability and translation may depend on the balance between the different proteins bound.[46–49]

RBPs are implicated in a number of human diseases, such as cancers, metabolic disorders, neuropathies, and muscular atrophies.[50–52] These proteins are characterized by a multitude of functional domains and sequence motifs, including the RNA-binding domain (RBD) or RNA recognition motif (RRM), K-Homology domain, cold shock domain, RGG (Arg-Gly-Gly) box, Sm domain, zinc-finger domain, double-stranded RRM, PUF repeat, and arginine rich motif (reviewed in Refs 53-55). However, many RBPs, especially those with multiple cellular functions, do not possess these motifs and have been identified as noncanonical RBPs,[56-59] as is the case for GAPDH. Other metabolic enzymes, including aconitase,[60] aldolase,[3] lactate dehydrogenase (LDH),[43] phosphoglycerate kinase (PGK),[61] glucose-6-

phosphate dehydrogenase (G6PDH),[62] glutamate dehydrogenase (GDH),[63] and isocitrate dehydrogen-ase (IDH)[64] have been shown to play similar roles (reviewed in Ref 65). For these noncanonical RBPs, little is known about their RNA-binding sites and the mechanisms allowing these enzymes to switch between their metabolic and RNA-binding functions.

GAPDH–RNA INTERACTIONS

Transfer RNA

Nuclear export of tRNAs into the cytoplasm is a carrier-mediated process,[66] and GAPDH has been shown to be involved in tRNA translocation, in agreement with its nuclear and cytoplasmic localizations.[67] Several mutations and deletions in tRNA impaired GAPDH binding, thus demonstrating the key roles of the T-stem, anticodon, and variable loops in this specific interaction. Binding between GAPDH and *in vitro* transcribed *Escherichia coli* $tRNA^{tyr}$ and yeast $tRNA^{ser}$ occurred with high affinity (apparent K_D of 18 nM), and was disrupted by high concentrations of NAD^+, suggesting that the Rossman fold may be part of the tRNA-binding site.[67] Raman spectroscopy and circular dichroism characterization of GAPDH-$tRNA^{phe}$ complex also alluded to a potential role of the Rossman fold in the interaction (*vide infra*).[68]

Cellular RNAs

There are numerous reports of interactions between GAPDH and cellular RNAs, both *in vitro* and in cells (Table 1). Many of these studies rely on *in vitro* qualitative experiments including UV cross-linking, native PAGE, and electrophoretic mobility shift assays (EMSAs), but few report quantitative measurements of binding affinity, for example via fluorescence anisotropy. In cells, characterizations include co-immunoprecipitation, ribonucleoprotein immunoprecipitation, RNA-affinity pull-down, mRNA decay kinetics, and luciferase assays. Only a handful of these studies characterized the effect of GAPDH binding on the RNA structure. The direct biological consequences of many of these interactions have yet to be elucidated. Regardless, the diversity of the RNAs interacting with GAPDH allows for a greater appreciation of the intricacies of this complex enzyme in its cellular environment.

GAPDH is an AUBP—Nagy and Rigby were the first to demonstrate the proclivity of GAPDH binding to AU-rich RNA.[69] GAPDH was shown to bind AU-rich RNA probes derived from the 3′ UTR of interferon-γ (IFN-γ), c-myc, granulocyte macrophage colony-stimulating factor (GM-CSF), and interleukin-2 (IL-2) *in vitro*.[69] Other AU-rich mRNA targets of GAPDH include colony-stimulating factor-1 (CSF-1),[71] cyclooxygen-ase-2 (Cox-2),[73] connective tissue growth factor (CTGF/CCN-2),[74] endothelin-1 (ET-1),[75] and angiotensin II type I receptor (AT1R).[76] In our laboratory, we have recently shown that GAPDH binds to the core ARE from tumor necrosis factor-α (TNF-α) 3′ UTR mRNA *in vitro*.[77] We showed qualitatively (EMSA) and quantitatively (fluorescence anisotropy) that GAPDH binding to the ARE-probe occurred via a sequential two-step binding mechanism yielding a high-affinity complex (K_{D1} = 97 nM) and a low-affinity complex (K_{D2} = 1.4 μM). To our knowledge, this is the first identification of multiple ribonucleoprotein complexes between GAPDH and a cellular mRNA. While the ARE-RNA targets of GAPDH differ in

sequence and length, they all contain three to eight AUUUA pentanucleotide motifs, required for strong binding.[69] The biological relevance of some of these interactions was confirmed in cells, as discussed below.

(De)stabilization of mRNA Transcripts by GAPDH—GAPDH was found to modulate the stability of mRNA transcripts and regulate protein expression for genes important in cancer (CSF-1, Cox-2, CTGF/CCN-2), in cardiovascular homeostasis (ET-1), and in brain function (SCN1A). GAPDH binding to these mRNAs may either promote disease progression (CSF-1, CCN2), or alleviate disease conditions (Cox-2, ET-1, SCN1A), suggesting that targeting these interactions may have important therapeutic applications.

GAPDH was shown to be overexpressed in ovarian cancer cells and interact with the ARE within the 3′ UTR of CSF-1.[71] Small interfering RNA (siRNA)-mediated downregulation of GAPDH resulted in decreased stability of the mRNA transcript, concomitant with decreased CSF-1 mRNA and protein levels. These results suggested that GAPDH binding promoted CSF-1 mRNA stabilization and increased production of CSF-1 protein,[72] both of which are associated with progression or recurrence of the disease and poor prognosis. Similarly, GAPDH was shown to increase the stability of the CCN2 mRNA via binding to the *cis*-acting element of structure-anchored repression (CAESAR).[74] Hypoxia-induced gene expression of CCN2 is post-transcriptionally modulated by CAESAR, which is an ARE-destabilizing motif in the 3′ UTR of CCN2 mRNA.[80,81] In hypoxic conditions, GAPDH binding to CAESAR leads to CCN2 overexpression and may contribute to its key role in tumor angiogenesis and bone metastasis.

In contrast, GAPDH was shown to destabilize the mRNA of ET-1, Cox-2, and SCN1A. GAPDH associates *in vitro* and in cells with the 3′ UTR of ET-1 mRNA.[75] Partial knockdown of GAPDH via siRNA treatment resulted in increased ET-1 mRNA and protein levels in cells and a modest increase in reporter expression, suggesting that GAPDH mediates the 3′ UTR-dependent downregulation of ET-1 mRNA.[82] Notably, ET-1 mRNA half-lives were indirectly determined from luciferase activity, which also depends on translational control. Thus, the exact mechanism by which GAPDH regulates ET-1 expression may need further examination. Regardless, as increased ET-1 levels are associated with many diseases,[83,84] controlling ET-1 production via modulation of its mRNA stability offers a promising novel therapeutic strategy.[85] Similarly, siRNA-mediated knockdown of GAPDH lead to a moderate increase in Cox-2 mRNA and protein levels after lipopolysac-charide induction, suggesting that GAPDH may destabilize the Cox-2 mRNA transcript.[73] Native PAGE gel-shift assays suggested that GAPDH binding to the core ARE of the Cox-2 mRNA 3′ UTR may be regulated by cellular GSH/GSSG ratios and GAPDH localization, however, the biological consequences of this interaction remain to be determined. Finally, in neurons, the voltage-gated sodium channel type 1 a subunit (NaV 1.1) is encoded by the *SCN1A* gene. Mutations within this gene have been linked to migraine, epilepsy, autism, and Dravet syndrome.[86] The C1794U mutation in the 3′ UTR of SCN1A mRNA was shown to affect protein expression and mRNA stability, possibly via changes in predicted mRNA secondary structures. GAPDH was shown to selectively bind to the U-rich mutant 3′ UTR and to negatively regulate protein expression and mRNA stability.[78]

Translational Regulation by GAPDH—In two cases, GAPDH was found to regulate protein expression at the translational level and not by affecting mRNA stability. GAPDH was found to be a key mediator in the enhancement of effector function of activated T cells via aerobic glycolysis by inhibiting the translation of IFN-γ and IL-2.[70] In activated T cells deprived of aerobic glycolysis, GAPDH immu-noprecipitation showed its association with the ARE of the IFN-γ mRNA 3′ UTR. GAPDH binding inhibited IFN-γ protein production and subsequent effector function in T cells.[70] These results further suggested that GAPDH may switch between its various functions depending on the energetic status of the cell *(vide infra).* Similarly, GAPDH was shown to regulate the translation of AT1R, which plays a crucial role in cardiovascular homeostasis. GAPDH binds the AT1R 3′ UTR *in vitro* and in cells,[76] and decreases AT1R expression by inhibition of AT1R translation. In addition, GAPDH mediates the effects of oxidative stress on AT1R expression via its decreased expression and binding affinity to the AT1R 3′ UTR.

Importantly, for both IFN-γ and AT1R, regulating the levels of GAPDH may enhance the efficacy of immunotherapy treatments, help control aberrant inflammation, and restore healthy levels of AT1R after oxidative stress.

Additional Interactions—GAPDH was found to bind the hammerhead ribozyme directed against tumor necrosis factor-α (TNF-α-Rz) in peripheral blood mononuclear extracts. Binding of GAPDH to TNF-α-Rz was much stronger than binding to tRNA or ARE-probes and was inhibited by the NAD^+ cofactor.[79] This interaction protected TNF-α-Rz from cellular degradation, and enhanced its cleavage activity possibly due to GAPDH unfolding activity.

Under stress, GAPDH has been shown to travel to the nucleus to act as either a protective[12,13] or apoptotic agent.[7,8,23-25] In the nucleus of noncancerous cells, GAPDH is involved in the maintenance of telomeres, functioning in antiaging and cell protective capacities.[12,13] In contrast, in breast cancer cells overexpressing GAPDH, cell senescence occurred via GAPDH inhibition of telomerase.[14] GAPDH binding to the telomerase RNA component (TERC) was thought to occur via the Rossman fold, but did not directly cause inhibition. Instead, TERC binding promoted inhibition of the telomerase reverse transcriptase (TERT) by the GAPDH C-terminal domain.[14] These results demonstrate that multiple regions of the protein can be utilized in tandem to perform specific tasks.

GAPDH was also shown to interact with the 3′ UTR of myosin heavy chain (MyHC) mRNA, mostly in skeletal muscle cells.[3] The role of this interaction has not been established, although it was proposed that it may serve a localization function, as GAPDH is also known to interact with both microtubules[2] and actin filaments.[87]

It is intriguing that GAPDH binding to various cellular mRNAs can result in either stabilization or destabilization of the specific target. A structure–function model has been proposed to explain this apparent discrepancy, whereby the catalytic domain may play an active role in RNA binding.[19] In addition, several pathways have been described for the posttranscriptional control of gene expression.[49] However, the exact mechanisms by which GAPDH affects mRNA stability and/or protein expression remain unknown. The dual role of

GAPDH in regulating mRNA decay is reminiscent of that played by the well-established AUBP, AUF-1.[88] It has been proposed that the fate of mRNA depends on the balance between stabilizing and destabilizing AUBPs. This mechanism is very attractive for GAPDH, as other AUBPs bind to the same AREs.[46,72]

Viral RNAs

GAPDH has been shown to either protect cells against viral infection or to promote virus progression via interaction with the viral genome. While cells possess intrinsic antiviral properties via host protein-viral nucleic acid interactions, many viruses are also known to co-opt host cell factors to assist in gene expression and replication (Table 2).

Antiviral Functions of GAPDH—The means by which GAPDH can function in a cell protective capacity varies depending on the specific virus. The majority of RNA viruses with which GAPDH interacts have single-stranded positive sense genomes, which replicate via asymmetric synthesis of positive sense progeny RNA.[101] GAPDH shows preferential binding to the 3′ UTR of positive sense progeny RNA of both the bamboo mosaic virus (BaMV) and its satellite virus satBaMV, reducing the positive to negative ratio and diminishing viral propaga-tion.[89] Studies with GAPDH knockdown in *Nicotiana benthamiana* showed that GAPDH binding to positive sense progeny RNA prevents the transcription of negative sense RNA and halts the replication process.[89]

Additional GAPDH antiviral functions arise from competing with other cellular proteins for RNA binding as seen with the hepatitis A virus (HAV). GAPDH has been found to bind to its 3′ UTR[90] and its 5′ UTR and compete with the polypyrimidine tract-binding protein (PTB).[91-93] While PTB binding increased gene expression, GAPDH binding decreased gene expression, notably through its ability to destabilize the RNA secondary structure.[92] In the case of transmissible gastroenteritis virus (TGEV), siRNA-mediated gene silencing showed a threefold increase in replicon activity following GAPDH knockdown.[94] It is not clear whether GAPDH's protective role arises from competitive binding with other host proteins, including PTB, or preferential binding of the positive sense progeny RNA as seen with BaMV.

Viral Subversion of GAPDH—Many RNA viruses co-opt GAPDH for use in viral progression such as the positive sense single-stranded RNA (ssRNA) hepatitis C virus (HCV),[95] Japanese encephalitis virus (JEV),[96] and tomato bushy stunt virus (TBSV),[97,98] and the negative sense ssRNA hepatitis D virus (HDV),[99] and human parainfluenza virus (HPIV).[100]

In the case of positive sense ssRNA viruses, GAPDH can be co-opted to either stabilize the viral RNA or alter the positive:negative progeny ratio. During HCV infection, GAPDH stabilizes the viral RNA via binding to its 3′ UTR.[95] For JEV infection, GAPDH was shown to preferentially bind negative sense RNA, but the biological consequences of the interaction remain unclear.[96] For TBSV, GAPDH was shown to sequester negative sense RNA to the replicase complex and promote the release of positive sense progeny to support viral replication.[97,98]

HDV is a satellite of Hepatitis B virus and one of two negative sense ssRNA viruses known to bind GAPDH via the UC-rich regions of the genomic and antigenomic RNA.[99] As seen with TNF-α ribozyme, GAPDH binding was observed to increase the intrinsic ribozyme activity of the HDV RNA.[99] Finally, GAPDH was shown to bind the 3′ UTR of the genomic sequence of HPIV3 *in vitro* and in cells.[100] For many of the GAPDH-viral RNA interactions, including BaMV, HAV, JEV, and HDV, several ribonucleoprotein complexes were observed by EMSA. As in the case of GAPDH-TNF-α ARE interactions,[77] the biological significance of these multicomplexes has not yet been elucidated.

GAPDH: A MISSING LINK BETWEEN RNA BIOGENESIS AND ENERGY METABOLISM?

Evidence has emerged to further delineate the complexities of the involvement of GAPDH and other metabolic enzymes in the regulation of RNA translation. The REM (RNA, Enzyme, Metabolite) hypothesis notes that many intermediary metabolic enzymes possess RNA-binding functions. As such, there may be posttranscriptional regulatory networks that exist between metabolism and gene expression based on RNA, enzyme, and metabolite interactions.[102] The first example of a link between RNA biogenesis and intermediary metabolism came from the finding that SRN1, a suppressor of RNA processing in yeast, relieves glucose repression.[103] Such links have also been made with GAPDH binding to the glucose transporter, GLUT-1, mRNA. The expression of GLUT-1 is regulated via alterations in mRNA turnover following treatment with TNF-α.[104,105] GAPDH was shown to bind to the AU-rich region of the GLUT-1 mRNA 3′ UTR *in vitro*.[62] While the biological consequences of this interaction have yet to be determined, it is tempting to speculate that GAPDH could regulate the expression of GLUT-1 in response to nutrient levels (Figure 1). This hypothesis is reinforced by the fact that GAPDH was previously shown to regulate the function of mTOR as a function of glycolytic flux. Under low glucose conditions, GAPDH inhibited $mTOR_{C1}$ by binding to and sequestering the GTPase Rheb, preventing cell growth and proliferation.[106]

Several other lines of evidence support the hypothesized link between metabolism and mRNA binding by GAPDH, including the fact that ketogenic diets (high in fat, low in carbohydrates, shifting metabolism from glycolysis to ketosis) reduce the effects of Dravet syndrome and epilepsy. GAPDH was shown to bind mutant SCN1A mRNA and decrease the translation of the sodium transporter NaV 1.1 *(vide supra)*.[78] It may be that in a ketogenic environment, GAPDH would be free from the demands of glycolysis and could more readily bind to SCN1A transcripts and alter their translation (Figure 1). This hypothesis remains to be tested.

Many normal cells as well as tumor cells preferentially utilize glycolysis over oxidative phosphoryla-tion even under normoxic conditions. This could be due to the increased energy demand of highly proliferating cells, but it could also have additional utilities. Indeed, activated T cells rely on aerobic glycolysis to obtain full effector status via mRNA translation of IFN-γ and IL-2 cytokines *(vide supra)*. In cells not relying on glycolysis, GAPDH binding to IFN-γ and IL-2 mRNA increased and correlated with decreased

cytokine production, suggesting that GAPDH binding to the AU-rich 3′ UTR of either mRNA could prevent their translation.[70] It is thought that in cells relying on glycolysis or in cells deprived of nutrients by neighboring cancer cells, GAPDH cannot bind to and inhibit cytokine translation. Addition of glucose reverses this effect. In this way, GAPDH would serve as a metabolic sensor to coordinate efficient glycolysis to T cell function[70] (Figure 1). This hypothesis requires further examination, as it may provide additional means to avoid aberrant inflammation or to restore T cell effector function.

In summary, GAPDH (and other metabolic enzymes) could provide the missing link between nutrient sensing and regulation of gene expression, crucial to several cellular pathways. However, more studies are required to confirm this hypothesis.

STRUCTURAL DETERMINANTS OF GAPDH

Human GAPDH is expressed as a 36 kDa polypeptide of 335 amino acids that can be divided into two domains: a Rossman fold/cofactor-binding domain (residues 1–150 and 317–335) and a catalytic domain (residues 151–316; Figure 2(a)). The protein is highly conserved across all kingdoms of life and its enzymatic function requires a 144 kDa homotetramer that is best described as a dimer of dimers.[107] The four subunits are labeled O, P, Q, and R with the pairs O/P and Q/R each forming a dimer (Figure 2 (b)). The identical subunits are structurally very similar to each other [average root mean squared deviation (RMSD) <0.3 Å] and are related to each other by three twofold symmetry axes *P, Q,* and *R*. The dimer interfaces are located along the *P* axis, while the tetra-mer interface (between dimers O/P and Q/R) is located along the *R* axis. The catalytic domains form the dimer interface via a five-stranded antiparallel β-sheet (Figure 2(c)), while the NAD+-binding domains participate in the tetrameric interface.

The Catalytic Domain and Putative RRM

We performed a structural alignment[108] between GAPDH (PDB code 4WNC) and the RRM1 from HnRNP L (PDB code 3R27), yielding an overall RMSD of 3.7 Å for 78 amino acids (our unpublished data and Figure 3). The RRM-like structural motif in GAPDH encompasses residues 152–178, 242–267, 274–277, and 291–315, and adopts a similar three-dimensional structure to the canonical RRM (βαββαβ fold), but shares no sequence similarity. Interestingly, GAPDH residues 303–308 were previously proposed as a putative tRNA-binding site.[68] The RRM-like structural motif in GAPDH is located at the dimer interface, but is not positively charged or rich in aromatic residues, as seen for other RBPs. Because RRMs can also mediate protein–protein interactions and have been found in proteins that do not bind RNA,[109] it remains to be seen whether this motif is involved in GAPDH binding to RNA *(vide infra)*.

NAD^+-Binding Site and Oligomeric Interface

The structures of GAPDH from archaea, bacteria, and eukaryotes have been determined by x-ray crystallography, and show that the NAD+ cofactor is bound, despite not being added during protein purification. In mammalian GAPDH structures, two,[110] three,[77,107] or even four NAD^+ molecules have been observed[111-113] per homotetramer. In addition, we recently

showed that GAPDH was present as a mixture of NAD^+-bound and NAD^+-free species in solution by nano-ESI/MS/MS studies.[77] These results confirm that GAPDH binds NAD^+ with high affinity, and that studies aiming at determining the effect of the cofactor should be done with NAD^+-free GAPDH.

Our recent hydrogen–deuterium exchange mass spectrometry studies showed an unexpected connection between the NAD^+-binding site and the dimer interface.[77] A single mutation at the dimer interface promoted short-range and long-range structural changes and increased dynamics of protein regions at the dimer and tetramer interfaces, and in the distant NAD^+-binding site (Figure 4). In addition, the mutation significantly decreased the amount of NAD^+-bound GAPDH species present in solution. These results strongly suggest an unexpected link between GAPDH oligomeric interfaces and the cofactor-binding site.

THE ELUSIVE SEARCH FOR THE GAPDH RNA-BINDING SITE

GAPDH is undoubtedly an RBP, but it does not contain any canonical RNA-binding sequences. Despite over 30 years of research, the RNA-binding site remains elusive. Several GAPDH regions have been proposed as putative RNA-binding sites, including the Rossman fold, the positively charged substrate-binding groove, and the dimer interface.

The Rossman Fold

The Rossman fold, which binds NAD^+, has often been heralded as the putative RNA-binding site of GAPDH (Figure 5(a)). Several lines of evidence support this hypothesis. A multitude of other metabolic enzymes also bind RNA, several of which are NAD^+-dependent dehydrogenases with similar mononucleotide-binding domain 1 in the Rossman fold.[115] The majority of studies discussed herein employ competition experiments demonstrating the ability of cofactor and substrate to decrease RNA binding in a concentration-dependent manner, implying that a shared binding site exists. This phenomenon has been observed with all RNAs shown to bind to GAPDH,[67,69,73-75,79,89,90,92,115] except in one case where increasing NAD^+ concentrations increased binding of GAPDH to *ccn2* mRNA.[74] However, many of these studies rely on UV cross-linking of the ribonucleoprotein complexes prior to EMSA.[14,69,73,74,115] Because NAD^+, NADH, and ATP strongly absorb at 260 nm, increasing concentrations of these molecules would dramatically reduce the cross-linking efficiency. As a result, the observed decreases in GAPDH-RNA complexes could be artifactual, at least in some of these cases. Other reports showed a similar trend, even in the absence of UV cross-linking,[67,75,79] supporting a potential involvement of the Rossman fold in binding the RNA. Nevertheless, all studies used a large excess of NAD^+ compared to protein and RNA to observe a significant effect on RNA binding, thus suggesting that the cofactor-binding site may only be part of the RNA-binding site. In addition, in light of our results suggesting a connection between the NAD^+-binding site and the GAPDH-oligomeric interfaces,[77] these studies would be unable to distinguish between a direct competition or an allosteric effect by NAD^+.

To narrow down the RNA-binding sites, several groups have used truncated or mutated GAPDH constructs to perform RNA-EMSAs, with different outcomes. Full length and GST-$GAPDH_{1-151}$ were shown to interact with TERC, while the C-terminal construct GST-

$GAPDH_{148-335}$ did not.[14] Similar results were obtained with the AU-rich IFN-γ 3′ UTR, where GST-$GAPDH_{1-43}$ was shown to be sufficient for binding.[69,115] In contrast, only full-length GAPDH was shown to bind to the AU-rich ET-1 3′ UTR.[75] Studies with mutant GAPDH (D35A, Y45A, S51G) confirmed a potential role of the Rossman fold in TERC binding.[14] Some of these mutations are key to the oligomeric interfaces, but any effects of the mutations on the structure of the protein were not discussed. In our laboratory, we have observed that several mutations, not directly involved in cofactor binding but present at the oligomeric interfaces, negatively impact GAPDH stability (unpublished data). Thus, RNA-binding activity of GAPDH truncations and mutations that may also affect protein stability, folding, oligomeric assembly, and activity, should be evaluated carefully.

Other NAD^+-dependent metabolic enzymes can bind RNA as well,[102,116] and some, including LDH, malate dehydrogenase, alcohol dehydrogenase, and G6PDH show similar sequence specificity for AREs in the mRNA 3′ UTR.[43,62,104] This suggests that the Rossman fold could serve as a noncanonical RNA-binding site. Recent studies have shown that this fold was underrepresented in the identification of the mRNA interactome in proliferating HeLa cells, possibly because their metabolic status may prevent Rossman-containing proteins to lectively, these results suggest that while the Rossman fold clearly plays a *role* in RNA binding, its importance may be dependent upon the specific RNA, cell type, and metabolic state.

The Positive Substrate-Binding Groove

GAPDH contains two regions spanning about 70 Å forming the substrate-binding grooves, which are located along the homotetramer *P* axis and are rich in positively charged amino acids (Figure 5(b)). These positive grooves have been suggested as the sites for RNA (and DNA) binding,[13,68] as they are wide enough (~25 Å) to accommodate an 18 Å-wide nucleic acid helix or loop.[68] In general, protein–RNA-binding interactions include hydrophobic stacking (affinity), electrostatic interactions (specificity), and hydrogen bonds, as seen in most RNA-binding motifs.[53] Aromatic residues, positively charged residues (Arg, Lys), polar residues (Asn, Gln), and negatively charged residues (Asp, Glu) are usually involved in protein interactions with ssRNA,[55] while proteins that bind double-stranded DNA (dsDNA) or double-stranded RNA (dsRNA) usually contains extensive positively charged regions on their surface.[55] It is not known yet whether GAPDH binds to ssRNA and/or dsRNA, but earlier studies suggested a preference for single-over double-stranded RNA.[29] Notably, single-stranded AU-rich RNA generally adopts specific secondary structures that GAPDH could preferentially bind to,[34,75,90] while dsRNA may contain specific nucleotide sequences important for GAPDH recognition. Finally, several studies have shown that the GAPDH substrate (G3P) competes with RNA binding,[14,70,75] further supporting a role of the positive grooves in RNA binding.

The Dimer Interface

The dimer interface of GAPDH has also been implicated in RNA binding (Figure 5(c)). Several lines of evidence support this hypothesis. First, it contains basic and aromatic residues (K227, R230, H305, F306). Second, the dimer-interface peptide 250–258 from yeast GAPDH (252–260 in human GAPDH) was cross-linked to an AU dinucleotide in cells

as shown by mass spectrometry.[114] This peptide is adjacent to residues 306–311, proposed to share sequence similarity to the RRM2 RBD,[68] and is also located along the positive groove. Third, we have shown that a dimer-interface mutant impaired formation of complex 2 between GAPDH and the TNF-α ARE probe, and affected the structure of bound RNA.[77] While more studies are needed to confirm whether the effect of the mutation is direct or indirect, our results suggested an involvement of the dimer interface in RNA binding.

Collectively, these studies suggest that the RNA-binding site in GAPDH would span a large area via a combination of the Rossman fold, the positive groove, and the dimer interface. This hypothesis is strongly supported by the interconnection of these regions in GAPDH.[77] In addition, human GAPDH requires at least three AUUUA pentanucleotides for strong binding,[69] supporting the hypothesis for an extended RNA-binding site in GAPDH. This is in contrast with the canonical RBDs that bind to shorter RNA sequences (2–8 nucleotides). However, many RBPs combine several RNA-binding motifs to bind longer RNA sequences with high affinity.[55,109] Similarly, GAPDH could combine several protein regions to achieve high affinity for RNA binding. Structural studies will be required to unambiguously elucidate the RNA-binding site in GAPDH and the exact stoichiometry of the ribonucleoprotein complex.

POSTTRANSLATIONAL CONTROL OF GAPDH BINDING TO RNA

Posttranslational modifications would offer an elegant mechanism to allow GAPDH to switch between its metabolic and RNA-binding functions. Many studies have linked GAPDH redox sensitivity via the active site cysteine (C152) to regulate its RNA-related functions. For instance, binding of tRNA,[69] IFN-γ,[69] and CTGF/CCN2[74] mRNAs was increased in the presence of 2-mercaptoethanol and decreased in the presence of diamide. In addition, glutathionylation was shown to inhibit GAPDH binding to the 3′ UTRs of Cox-2[73] and ET-1,[75] and cysteine oxidation reduced binding to TERC[14] and AT1R mRNA,[76] but increased binding to tRNA and DNA.[26] These results suggest that the GAPDH redox state affects nucleic acid binding. However, studies evaluating possible structural consequences of such modifications that could alter RNA binding are needed, as GAPDH oxidation was shown to lead to disulfide-linked aggregation.[118-120]

The roles of other posttranslational modifications of GAPDH in its RNA-binding function have not yet been elucidated. Many of these modifications occur at or near the dimer interface, including acetylation,[121,122] phosphorylation,[123,124] and glyco-sylation.[125,126] Thus, studies to determine whether these modifications affect GAPDH binding to RNA *in vitro* and in cells will be crucial. These modifications would offer GAPDH an elegant mechanism for switching between its various cellular functions as a function of the cell cycle and metabolic state.

In addition to posttranslational modifications, GAPDH switching between its glycolytic and RNA-binding functions could be promoted by its cellular localization, oligomerization state, interactions with other proteins, nutrient concentration (e.g., glucose), and redox and metabolic cell status. More research is necessary to elucidate the regulatory mechanisms

allowing this abundant enzyme to perform the multitude of cellular functions described so far (see Introduction).

GAPDH–RNA INTERACTIONS ARE CONSERVED IN OTHER SPECIES

The GAPDH amino-acid sequence is highly conserved across all species. The key role of GAPDH in the regulation of gene expression via RNA binding was also observed in plants and yeast. In plants, cytoplasmic GAPDH was shown to regulate replication of phytopathogenic viruses by binding to their RNA *(vide supra).* In yeast, several GAPDH isoforms (GAPDH1-3) are present (65% sequence identity with human GAPDH) and all three were shown to bind AU-rich RNA. Early studies showed that the *Saccharomyces cerevisiae* basic GAPDH isoform (*Sc* GAPDH1) bound to poly(U) RNA and had a strong nucleic acid-helix destabilizing effect.[29] The *Sc* GAPDH3 protein was shown to weakly bind AU-rich or Poly(A) RNA *in vitro* (K_D of ~110–150 μM), but not Poly(U).[127] In contrast to mammalian GAPDH, *Sc* GAPDH3 showed higher affinity for shorter RNA with only one or two AUUUA motifs. In yeast cells, the GAPDH2 isoform was identified in a cross-link to an AU dinucleotide by mass spectrometry.[114] These results confirm that the RNA-binding function of GAPDH is conserved in other organisms.

CONCLUSION

GAPDH binds to a wide array of RNA molecules *in vitro* and in cells. Many of these interactions appear to be dependent upon cell type and metabolic and redox states. GAPDH-RNA interactions appear to have three distinct outcomes with respect to RNA fate: decreased transcript stability, increased transcript stability, or inhibited translation. The factors that determine these outcomes are not well understood, but may involve preventing or promoting interactions with endonucleases, competition with other RBPs and translation machinery, or binding to and acting on the RNA target itself. These interactions could then elicit a coordinated regulatory response that is intricately tied into the metabolic or redox state of the cell. The interacting RNAs identified thus far are most likely only a small subset of the number of interacting partners, as most studies to date have focused on the binding of one or a few specific RNAs or RNA fragments *in vitro*. A repository of GAPDH–RNA interactions (GAPDH-RNA interactome) in cells would provide a better understanding of the complexities of GAPDH function in health and disease as well as shed light on the link between RNA biogenesis and intermediary metabolism.

Additional information crucial to the complete understanding of GAPDH-RNA interactions includes the delineation of regulatory mechanisms and the effects of various GAPDH posttranslational modifications on RNA binding. Many of the RNA targets of GAPDH are also targets for canonical RBPs and microRNAs. Understanding the complex interplay between these competing interactions is key to elucidate the regulation of GAPDH-RNA interactions and how they dictate variable levels of posttranscriptional gene regulation.

Many key questions remain unanswered regarding specific GAPDH-RNA interactions, the effect of GAPDH binding on RNA fate and structure, and the stoichiometry of the GAPDH-RNA complex(es). To answer these questions, structural determination of a GAPDH-RNA

complex is crucial. With this information, it may be possible to design small molecules, peptides, or microRNAs that mimic or disrupt the interaction between GAPDH and target mRNAs. As GAPDH regulates the expression of several proteins key to pathological conditions, modulation of mRNA levels via GAPDH may offer alternative strategies in the treatment of various diseases.

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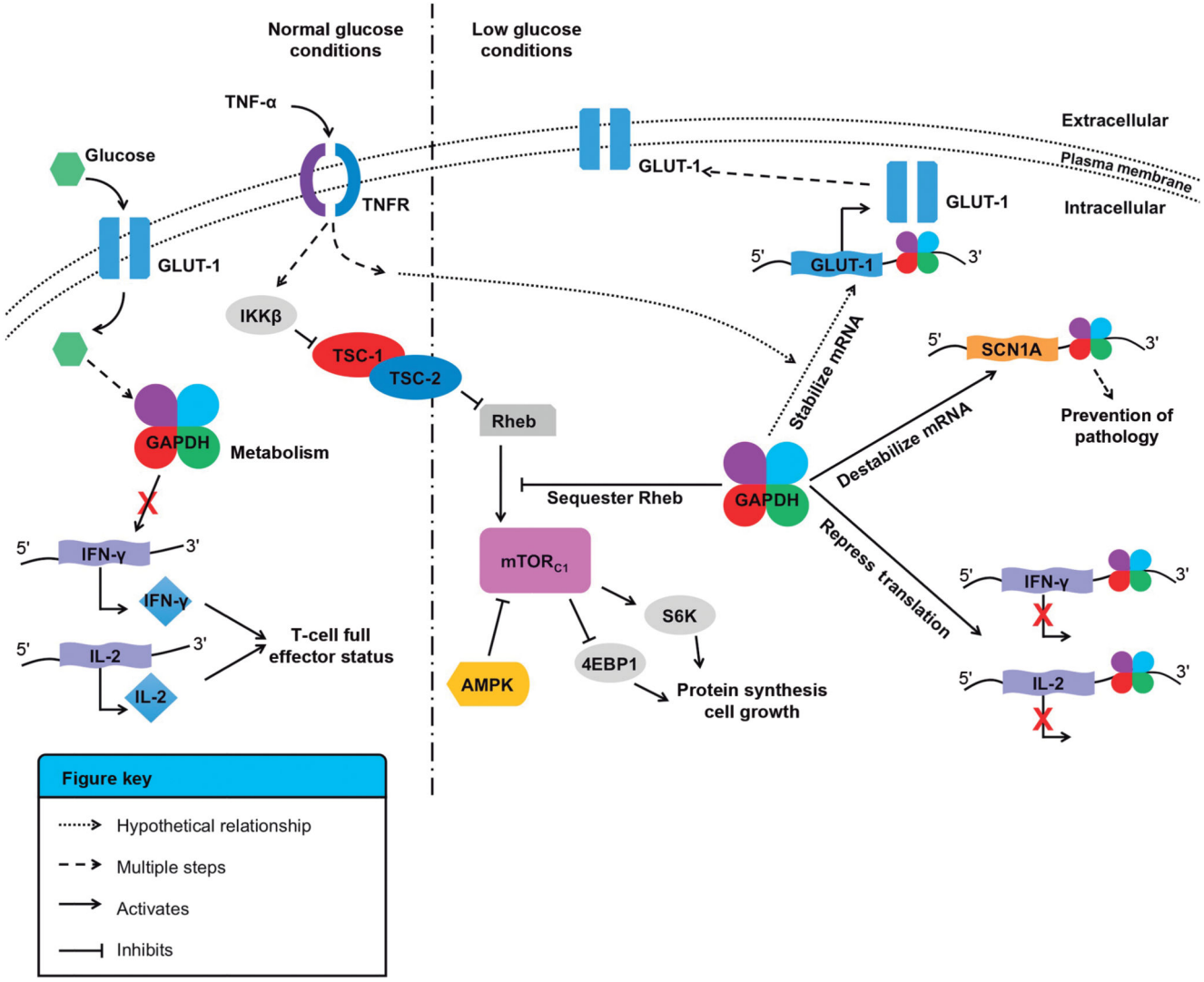

FIGURE 1.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), metabolism, and RNA binding. Schematic for the proposed links between GAPDH, metabolism, glucose sensing, and RNA binding. Under normal glucose conditions, GAPDH is involved in glycolysis. IKKβ mediates tumor necrosis factor-α (TNFα)-dependent inhibition of the tuberous sclerosis complex (TSC1-TSC2) via phosphorylation, decreases its activity toward the G-protein Rheb (Ras homolog enriched in the brain), and activates the mTOR (mammalian target of rapamycin) complex 1 (mTORc1), which leads to protein synthesis and cell growth.[106] Under low glucose conditions, GAPDH is involved in several pathways. First, GAPDH may stabilize the glucose transporter (GLUT-1) mRNA via binding to its AU-rich 3′ UTR.[62] Second, GAPDH binds to the 3′ UTR of interferon-γ (IFN-γ) and interleukin-2 (IL-2) and decreases translation of both cytokines, preventing activated T cells to reach full effector status.[70] Third, GAPDH sequesters Rheb and prevents mTORc1 activation.[106] Finally, in ketogenic diets, GAPDH may be free from glycolysis and able to destabilize the SCN1A

mRNA to reduce translation of the sodium transporter NaV 1.1, and alleviate conditions linked to Dravet syndrome.[78]

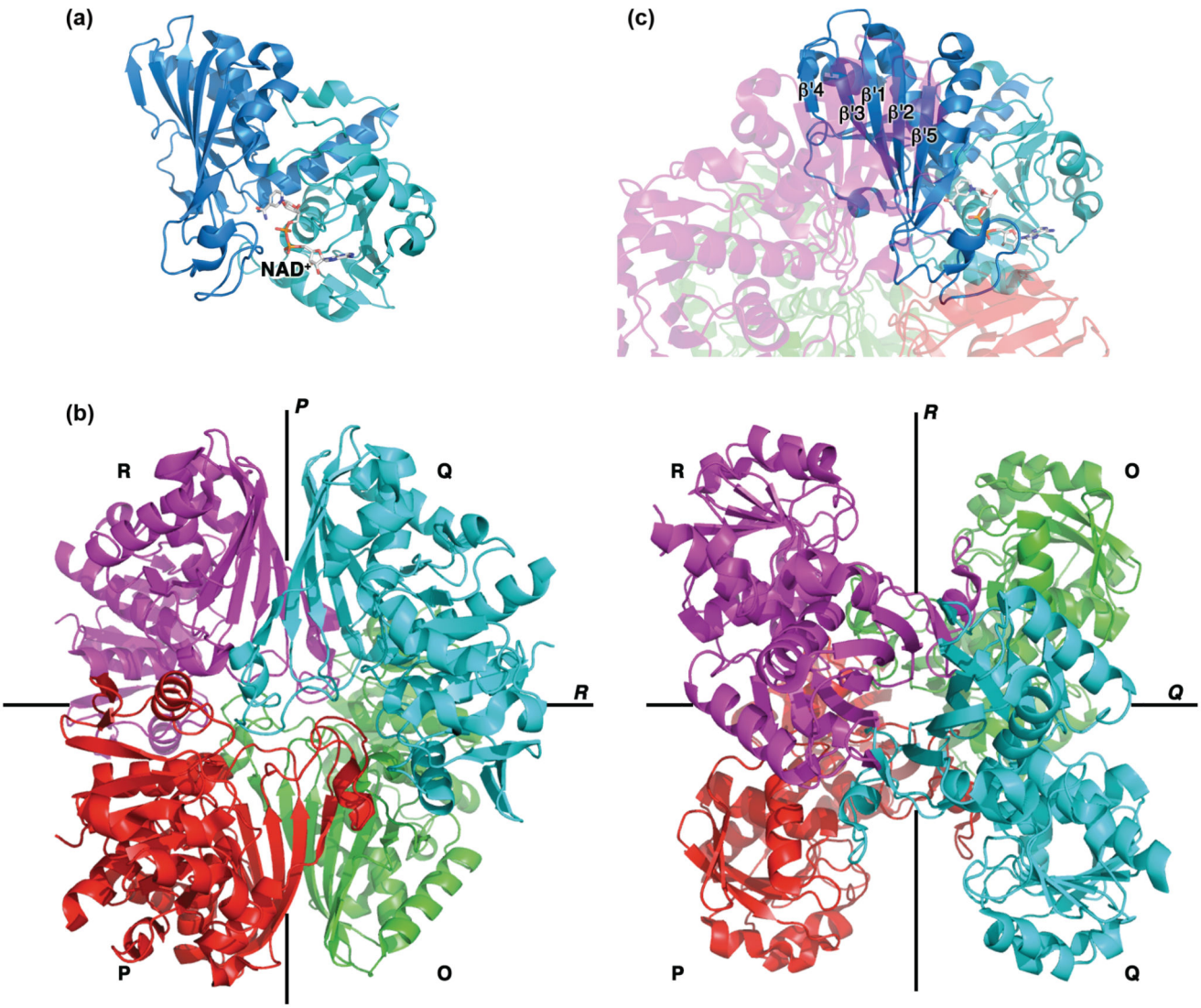

FIGURE 2.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) structural overview. (a) Each GAPDH subunit is comprised of two domains, the Rossman fold/cofactor-binding domain (cyan) and the catalytic domain (blue). (b) Tetrameric assembly of GAPDH (PDB code 4WNC). The four subunits (O-R) are related to one another by three twofold symmetry axes (*P*, *Q*, and *R*). (c) The dimer interface is formed by antiparallel five-stranded β-sheets from the R and Q (or P and O) subunits.

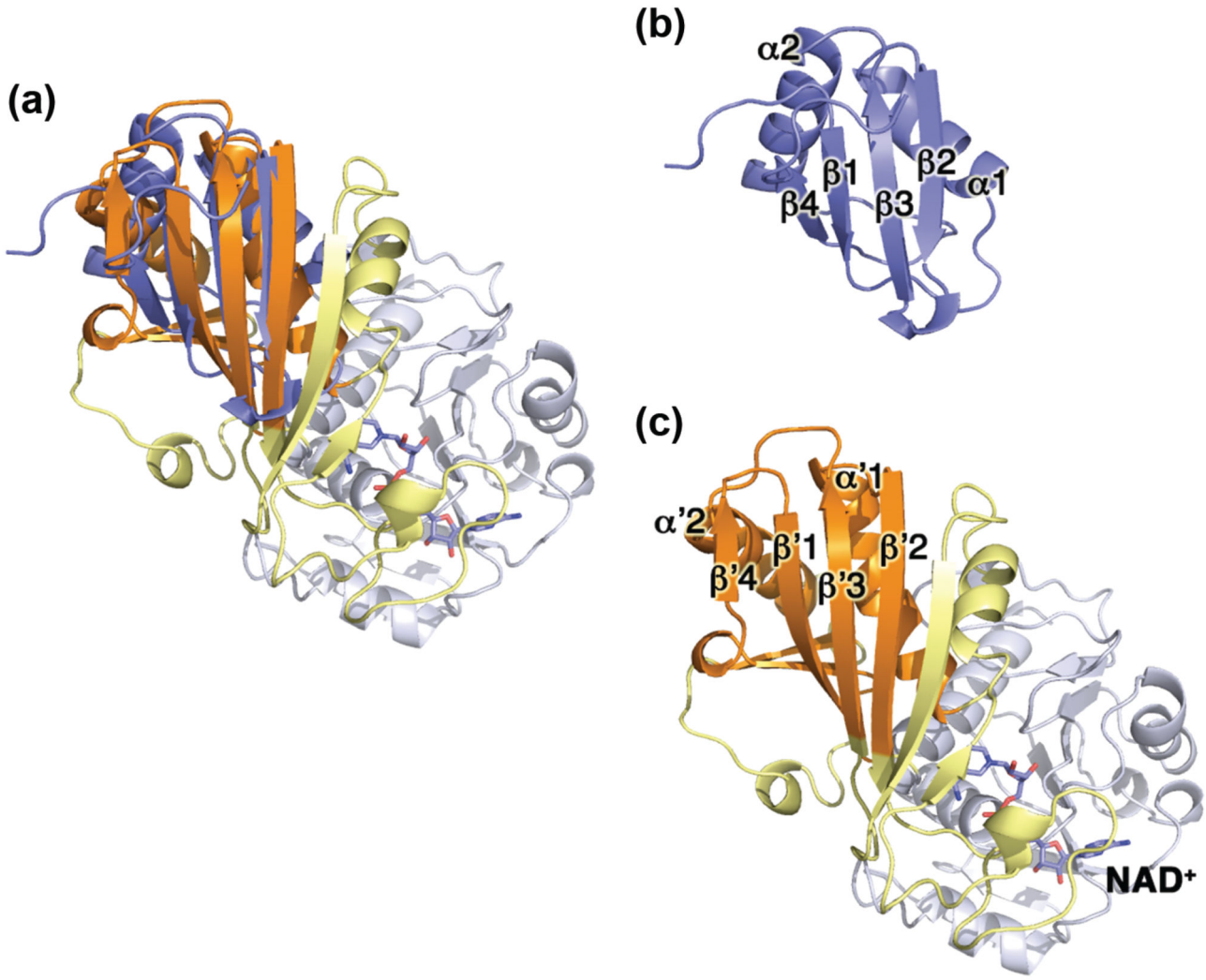

FIGURE 3.

Structural comparison of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and heterogeneous nuclear ribonucleoprotein L. (a) Structural overlay of monomeric GAPDH and HnRNP L (blue). The x-ray structures of HnRNP L and GAPDH are shown separately in (b) and (c) for clarity. (b) X-ray structure of the first RNA recognition motif (RRM) domain of HnRNP L (PDB code 3R27) showing the canonical βαββαβ motif. (c) Structure of the GAPDH monomer (PDB code 4WNC) showing the conserved structural motif (β′α′β′β′α′β′) in the catalytic domain. The NAD^+-binding domain of GAPDH is shown in white, the catalytic domain is shown in yellow and orange (RRM-like subdomain).

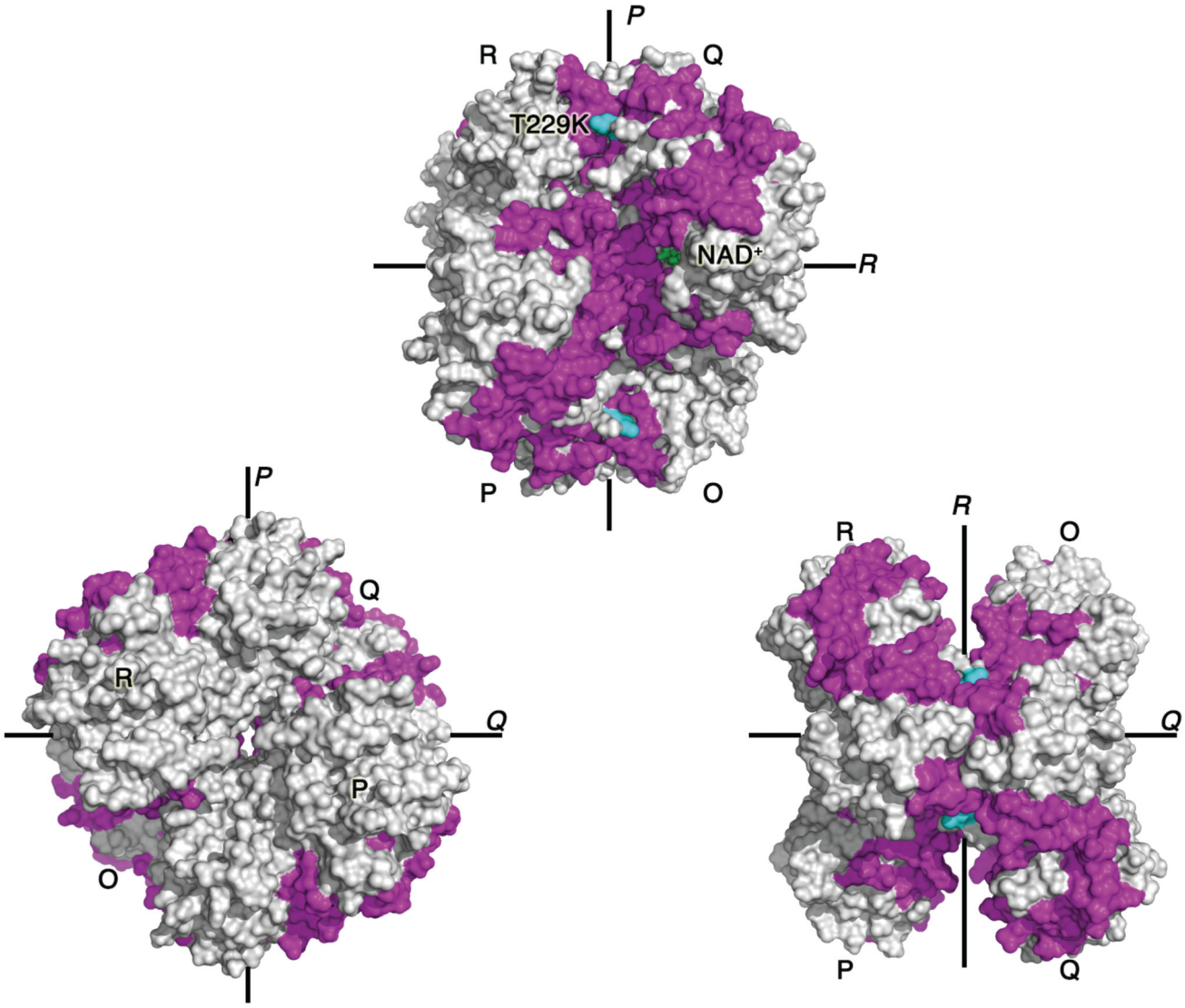

FIGURE 4.

Interconnection between oligomeric interfaces and NAD^+-binding site. Dimer-interface mutation T229K (cyan) induces a series of subtle conformational shifts that propagate throughout the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) tetramer. We showed that regions displaying increased solvent exchange upon mutation (magenta) are clustered along the *P* axis: dimer and tetramer interfaces, and NAD^+-binding site (green sticks).[77]

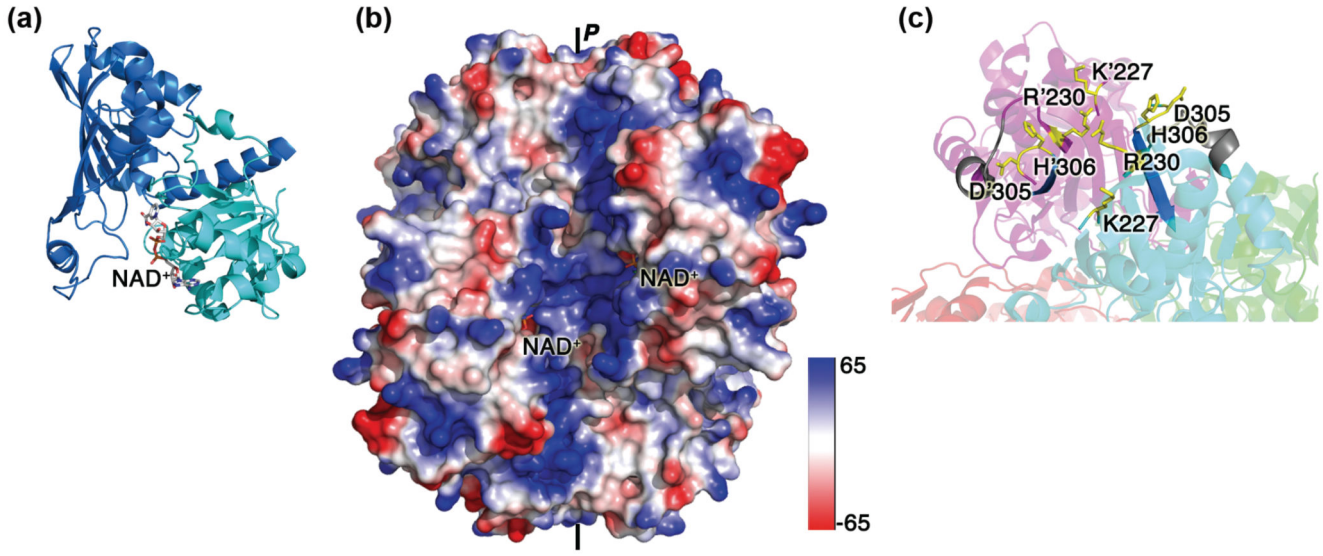

FIGURE 5.

Proposed RNA-binding sites in glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (a) Structure of the GAPDH monomer with the Rossman fold/NAD^+-binding domain (cyan) and the catalytic domain (blue). RNA was proposed to bind in or near the NAD^+-binding site. (b) Electrostatic potential of GAPDH mapped onto the solvent-accessible surface and colored by electrostatic potential with electropositive regions colored blue and electronegative regions colored red. The positively charged substrate grooves span the entire length of the GAPDH tetramer along the *P*-axis. (c) The dimer interface contains basic and aromatic residues that may play a role in RNA binding. Peptides that were proposed to be involved in RNA binding are highlighted in gray (peptide 252–260)[114] and dark blue (residues 306–311).[68]

TABLE 1

Cellular RNAs Shown to Interact with GAPDH

	RNA Stability		Protein Expression				
			Translational Efficiency		Abundance		
RNA	***Increase***	***Decrease***	***Increase***	***Decrease***	***Upregulated***	***Downregulated***	**Reference**
tRNA							67 – 69
IFN-γ				X		X	69,70
c-myc							69
GM-CSF							69
IL-2				X		X	69,70
CSF-1	X				X		71,72
Cox-2		X				X	73
CTGF/CCN-2	X				X		74
ET-1		?		?		X	75
AT1R				X		X	76
TNF-α							77
SCN1A		X				X	78
TNF-α ribozyme	X						79
TERC							14
MyHC							3
GLUT-1							62

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; tRNA, transfer RNA; IFN-γ, interferon-γ; GM-CSF, granulocyte macrophage colony-stimulating factor; IL-2, interleukin-2; CSF-1, colony-stimulating factor-1; Cox-2, cyclooxygenase-2; CTGF/CCN-2, connective tissue growth factor; ET-1, endothelin-1; AT1R, angiotensin II type I receptor; TNF-α, tumor necrosis factor-α; TERC, telomerase RNA component; MyHC, myosin heavy chain; GLUT-1, glucose transporter.

TABLE 2

GAPDH Interactions with Viral RNAs

Virus	Virus Family	Genetic Material	GAPDH Role	Reference
BaMV	Alphaflexiviridae	(+) ssRNA	Protective	89
satBaMV	BaMV satellite	(+) ssRNA	Protective	89
HAV	Picornaviridae	(+) ssRNA	Protective	90 – 93
TGEV	Coronoviridae	(+) ssRNA	Protective	94
HCV	Flaviviridae	(+) ssRNA	Co-opted	95
JEV	Flaviviridae	(+) ssRNA	Unknown	96
TBSV	Tombusviridae	(+) ssRNA	Co-opted	97,98
HDV	HBV satellite	(−) ssRNA	Co-opted	99
HPIV3	Paramoxyviridae	(−) ssRNA	Co-opted	100

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BaMV, bamboo mosaic virus; HAV, hepatitis A virus; TGEV, transmissible gastroenteritis virus; HCV, hepatitis C virus; JEV, Japanese encephalitis virus; TBSV, tomato bushy stunt virus; HDV, hepatitis D virus; HPIV, human parainfluenza virus; ssRNA, single-stranded RNA.