ACTIVATING OLIGOMERIZATION AS INTERMEDIATE LEVEL OF SIGNAL TRANSDUCTION: ANALYSIS OF PROTEIN-PROTEIN CONTACTS AND ACTIVE SITES IN SEVERAL GLYCOLYTIC ENZYMES

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1. ABSTRACT

A number of enzymes have inactive monomeric and active oligomeric forms. This suggests presence of definite interglobular contact -active site interaction in the enzymes. Although the phenomenon is widely studied *in vitro* as part of folding process the biological roles of the phenomenon, termed here as "activating oligomerization" are not clearly understood. In this work a procedure for analysis of protein-protein interactions was elaborated. Using spatial structures of several glycolytic enzymes potential role of kinase phosphorylation in regulation of oligomerization of the proteins as well as association of domains in a two-domain protein was assessed. In the enzymes 15-75% of kinase sites (mainly protein kinase C and casein kinase 2 sites) are placed in interglobular contact region(s). Upon being phosphorylated these sites may prevent oligomer formation. In structures of all the enzymes definite evidences of connection between active site and interglobular contact were found. Two structural mechanisms of interglobular contact influence on the active site were proposed. In addition to known mechanism of oligomerization initiated by allosteric metabolites the influence may be also exerted through functional sequence overlap and/or interdomain contact stabilization mechanisms. Implications for regulation of enzyme cellular function(s), signal transduction and metabolic analysis are considered. It is concluded that activating oligomerization may represent an intermediate level of enzyme cellular regulation.

2. INTRODUCTION

Formation of active oligomeric enzymes includes folding of the polypeptide chains and consecutive association of the chains. Association of the folded monomers may be studied using kinetic reconstitution experiments. During studies of this kind it was found that

Activating oligomerization

Table 1. Homo-oligomeric glycolytic enzymes used in analyses. Enzymes are listed in the sequence of glycolytic stages. TIM is enzyme of preparation phase of glycolysis, all others are enzymes of pay-off phase.

EC code	Enzyme name and abbreviator	Chain length	N glob	N dom.
5.3.1.1	Triosephosphate isomerase (TIM)	247	2	1
1.2.1.12	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	333	4	2
2.7.2.3	3-phosphoglycerate kinase (PGK)	416	1	2
5.4.2.1	Phosphoglycerate mutase (PGM)	246	4	1
4.2.1.11	Enolase (ENL)	436	2	2

many oligomeric enzymes have different catalytic properties of the monomeric and oligomeric forms: in the most cases, inactive monomer and active oligomer.

The process of quaternary structure formation is studied mainly on glycolytic enzymes, which are mostly oligomeric. It was shown that full enzymatic activity of some of them is achieved upon association (1). The same conclusion was drawn separately on the base of biochemical studies of glyceraldehyde 3-phosphate dehydrogenase (2), phosphoglycerate mutase (3) and enolase (4), analyzed in this work.

This process, here referred to as "activating oligomerization" is characteristic for many enzymes of glycolysis. What may be biological functions of this kind of oligomerization? Rate-determining step in triosephosphate isomerase folding at physiological pH seems to be the association of independently folded subunits (5)). The reconstitution of the yeast phosphoglycerate mutase indicated the rapid formation of structured monomers (with half time less than 10 s) is followed by more slow subunit association (6). Therefore, any change in the process of protein-protein association would lead to the change of cellular enzyme activity before all. Thus activating oligomerization may represent important stage of cellular enzyme regulation through the regulation of folding/assembly of the functional proteins.

Moreover, the three peculiarities of oligomerization: independent folding of subunits, subunit association as rate-limiting step and emergence of (full) activity upon association are also characteristic for some multidomain monomeric proteins. As example, analysis of the reversible unfolding of monomeric enzyme yeast phosphoglycerate kinase leads to the conclusion that the two domains are capable of folding independently (7), domain pairing has been suggested to be the rate-limiting step (8). Thus, two-domain monomeric proteins also may be subject to the regulated oligomerization.

The most universal conventional routes of cellular regulation are allosteric regulation (including metal ions) (bacterial phosphofructokinase (9)), and kinase phosphorylation. The biochemically observed phenomenon of activating oligomerization leads to the general conclusion, that in the oligomeric enzymes there is definite interaction of interglobular contact and active site. This interaction may be studied in details, if spatial structures of the enzymes are available. This work concentrates on potential role of kinase phosphorylation in regulation of oligomerization and mechanisms of interglobular contact/active site interaction.

3. MATERIALS AND METHODS

3.1. Protein data set

Five consecutive homo-oligomeric glycolytic enzymes with known spatial structures were used for analysis (table 1). Spatial structures were obtained from PDB (10). Four of them are yeast enzymes, D-glyceraldehyde-3-phosphate-dehydrogenase is from lobster (*Homarus Americanus*). Following kinase site types were used: protein kinase C (PKC), protein kinase A (PKA), casein kinase 2 (CK2), calmodulin-dependent protein kinase 2 (CAM2), tyrosine kinase (TyrK). Patterns of all the sites are presented in PROSITE (11), excluding CaM2 pattern, which was taken from PhosphoBase (12).

3.2. Programs used

For accomplishing the analysis several analytic procedures were necessary: identification of interglobular contact and active sites/ligand binding regions; identification of kinase sites and sites placed in the region of interglobular contact. Programs SURFC (Surface Calculation), SCANPS (Scan ProSite) and SQSOVR (Sequence Overlaps) used for calculation of surface accessibilities, sequence pattern analysis and identification of sequence overlaps respectively were described in the work (13). Surface accessibilities for sequences were calculated as sums of residue accessibilities. For calculating accessibilities of oligomer was used program MERGEPDB, that allows preparing of one PDB molecule coordinate set from coordinates of several PDB molecules. As most influential phosphorylation sites were taken those having zero hydroxyl accessibilities in oligomer while having non-zero in monomer. In the present work several programs for analysis of interactions between the residues/ligands of specified globules. Algorithms are described in the Discussion section of this article.

3.3. Finding interglobular interactions bond list

Program INTG (Inter Globular interactions) takes a PDB entry and identifiers of two globules as parameters and composes bond list for interface of the globules. Bond list includes hydrogen bonds and atomic hydrophobic contacts. If given globule identifiers are the same, and then intraglobular bond list is composed, otherwise interglobular. Program outputs three files: bond list in "internal" numeric format (.BDI), bond list in text bond list format and list of hydrogen bonds with their geometric parameters. Text output it made in PDB-like "bond list format", developed by the author:

```
PDBFILE 2ypi chains A B
BOND  65 ASN  10 A THR  75 B  HB ND2 OG1 STRAND   5  11 LOOP   63  79
BOND  61 LEU  13 A PHE  74 B  HP CD2 CD2 LOOP    11  16 LOOP   63  79
BOND  59 LEU  13 A GLY  72 B  HB O    N   LOOP   11  16 LOOP   63  79
.
.
.
END
```

Table 2. Overlaps of interglobular sequences with kinase site and active site sequences in the structure of yeast TIM (PDB entry 2YPI).

Sequence 1	Sequence 2	Surface accessibility, sq. A		
		Monomer	**Dimer**	**Hydroxyl (dimer)**
IPAB 95 102	PKC 96 98	23	23	1.6 (OG Ser 96)
IPAB 43 48	CK2 45 48	41	15	0.0 (OG1 Thr 45)
IPAB 10 17	PGA1 10 12			
IPAB 95 102	PGA1 95 95			
PKC 96 98	PGA1 95 95			
PKC 235 237	PGA1 232 232			

There are 5 PKC, 6 CK2, 1 CaM-2 sites, gap value is 3. Surface accessibility values are given for kinase site sequences.
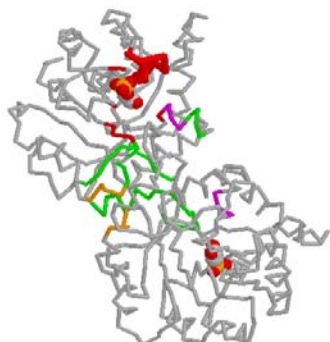
Figure 1. Interglobular contact, active site and related kinase sites in the dimeric molecule of yeast TIM. Ligand molecules are shown in space-filling mode for both globules, sequences of interglobular contact (green) and of product/ substrate binding (red) are shown for globule A. Enzyme's active signature sequence is emphasized. Two kinase sites in the interglobular region: CK2 site (orange) and PKC site (magenta) are shown for both globules.

First number in the BOND record is chain distance between contacting residues (applicable only for intraglobular contacts); "HB" or "HP" denotes hydrogen bond or hydrophobic atomic contact respectively. Interactions of bound ligands are listed in the same list for the given globules. If there are metal ion interactions, instead of metal atom name distance (in angstroms) between the ion and acceptor is given. Listing secondary structure elements for each bond record allows easy analysis of interactions between the secondary structure elements under interest.

3.4. Finding functional sequence on the base of bond list

Program BDISIT (Bond List- Sites list) converts the bond list file of interactions between two globules in "internal" numeric format (.BDI) into the list of sequences in .SIT format (13). Sequences were identified using given minimal gap value (default 3). Interglobular contact sequence, ligand sequences were extracted in a separate site type descriptor. If the sequences of the globules differ (heterodimer case), for residues of each globule a separate site type is made. In the case of homodimer interacting sequences from different globules are joined, as homodimer interglobular contacts are generally symmetric (unpublished data).

3.5. Joining information from several PDB experiments

Not any PDB entry may contain all the necessary data for analyses made in this work. Some of the entries may contain only one globule of oligomeric molecule and some other- only coordinates of bound ligand(s). Therefore, it was necessary to join the data from different PDB entries. In the case when all the data available for protein from one source, this was achieved simply by merging .SIT files. In the case when the sources are different (glyceraldehyde- 3-phosphate dehydrogenase in this work) necessary sequence data were transferred to the sequence under interest using pairwise sequence alignment. For preparation of alignments was used multiple alignment program Clustal W (14). Having correctly prepared alignment, the site sequences were transferred into the sequence under interest using elaborated program TRANSITE. The transposition of sequences represents much simpler and not less effective alternative to conventional 3D alignments or molecular-modeling techniques.

4. RESULTS

4.1. Yeast triosephosphate isomerase

As it is shown in the figure 1 and in the table 2, a PKC site is immediately involved in both active site and interglobular sequences. CK2 site overlaps only with an interglobular sequence. The accessibility of PKC site is not changed upon dimer formation, while accessibility of the CK2 site is reduced to zero (table 2) and the CK2 site modification may lead to significant sterichindrances during association of subunits. On other hand, PKC site also may lead to change in activity as it overlaps with one of the active site sequences (HIS 95, table 2). Although wild type monomeric subunits have some stability and residual catalytic activity dimerization causes a 1000-fold increase of $Kcat$ (15). There are two overlap regions of the interglobular contact and active site sequences. First of them is placed closer to N-terminus of each globule in the sequence IPAB 10-17, the second, site IPAB 95-102, also contains the interglobular PKC site PKC 96-98. Formation/dissociation of the active homodimeric molecule would inevitably affect interglobular region packing. Phosphorylation of interglobular kinase sites may disturb or even prevent oligomerization and enzyme's activity become affected due to partial folding/unfolding in the region of either or both functional overlaps with the interglobular contact sequences. Thus, regulation of TIM activity through interglobular kinase site modification is based on mechanism, termed here as "functional sequence overlap".

4.2.   Lobster   D-glyceraldehyde-3-phosphate dehydrogenase

No coordinates of NADp are presented in the PDB entry for lobster enzyme. As sequence of lobster protein shows 70-80% identity with $E.coli$ one, NADp-

Table 3. Kinase sites and ligand binding sequences in interglobular contact regions of lobster's GAPDH (PDB: 4GPD).

Kinase site/	PKC	CK2	CaM-2	TyrK
IP contact				
IP12 178 192	180 182	188 191		
IP13 275 277		275 277		
IP14 225 233			230 233	
238 244		237 240		244 251
276 281		273 276		
292 298		289 292		
309 309		308 311		
Per cent of sites	50%	75%	(100%)	(100%)

Kinase sites & ligand binding sequences

Sequence 1		Sequence 2	
PKC 317 319		NAD1 312 316	
CK2 99 102		NAD1 95 96	
CK2 308 311		NAD1 312 316	
IP14 200 204		SPS 207 208	

There are 4 PKC, 8 CK2, 1 TyrK, 1 CaM-2 sites. Interglobular contact 1-4 (dimer) is formed by the N-domain only, contact 1-3 (tetramer) by the C-domain, while contact 1-2 (tetramer) by both domains. As the molecule is homo-tetramer, the same is correct for each of the four subunits.

binding sequences were extracted from *E.coli* experiment using pairwise sequence alignment.   There is significant amount of kinase sites in the interglobular contact regions of GAPDH (table 3). Phosphorylation of some of them may influence formation of dimer and tetramer contacts with consequent impact on the activity and stability of the whole tetramer molecule.

No significant sequence overlaps were found in the structure of this enzyme. On one hand, a minor overlap of the dimer interglobular sequence set with NADp-binding was found in C-terminal region (NAD-binding: 312 316). As interglobular contact in this region is represented only by two atomic hydrophobic contacts of Trp 309, the activity hardly would be affected through this overlap. Monomeric glyceraldehyde 3-phosphate dehydrogenase is devoid of enzyme activity (2). On other hand, one of the substrate phosphate binding sequences (SPS 207 208) is placed near dimer contact sequence (IP14 200 204). The rest of the substrate (no data available) thus may be placed in a small cleft between two globules of the dimer (figure 2c) and represent interglobular binding site like that in bacterial phosphofructokinase (9). However, dimeric form is also inactive or has at least two orders of magnitude lower activity (16). Therefore, a mechanism other than functional sequence overlap or interglobular ligand binding must be implemented in the enzyme.

Two domains form each globule of the tetramer: N-domain (1-147) associated with C-terminal (316-333) and C-domain (148-315) (figure 2). NADp is bound by both domains (stretches 11-149, 312-316), residues from the second domain are also required for catalysis (Arg 195, Arg 231 (17)). This shows that maintenance of the interdomain contact of each subunit is essential for the catalytic activity.   Interglobular contact in dimeric form is formed only by the residues of the C-terminal domain (residues in range 225-309), whereas tetramer contacts are formed by the residues of both domains: 1-3 (41-45, 275-281), 1-2 (38-48, 178-234). Thus, interglobular tetramer contacts are stabilizing interdomain contact of each of the

four globules, as the molecule is homotetrameric (figure 2a-c).

4.3. Yeast phosphoglycerate kinase

Several kinase sites are placed relatively near (3-5 residues) to the interdomain contacting sequences (figure 3a). All of them have relatively high solvent accessibilities and possibly would have minor influence on the activity. Interdomain contact is also stabilized by interaction of the C-terminal fragment with the N-terminal domain (table 4). While hydroxyls of serines of the PKC sites have non-zero accessibility values, serine of CK2 is completely buried. Modification of this site may lead to partial disruption of the C-terminal fragment association with N-terminal domain.   There are two possible mechanisms of activation through domain association. On one hand, residues of the basic patch, required for catalysis (18) are placed exclusively in the N-domain, while all ligand binding sequences are in C-terminal one. On other hand, Mg- and 3-phosphoglycerate sequences are placed near interdomain contacting sequences (maximal overlap gap 5, table 4) and C-domain is stabilized by interaction with N-domain.

4.4. Yeast phosphoglycerate mutase

Several PDB entries were used for obtaining necessary set of data: 1BQ3- interglobular sequences; 3PGM- phosphoglycerate binding site sequences (table 5). Although no contacts of Arg 59 with ligand molecules were found using all available PDB experiments, Arg 59 is supposed to be involved in binding of phosphoglycerate (19).   Two PKC sites and one Tyr-kinase site has hydroxyls of potential phosphoresidues totally buried upon AB contact formation (table 5) that may influence formation as of dimer AB contact (PKC 28-30) as well as tetramer contacts. The 1st Tyr-phosphorylation site also overlaps with a phosphoglycerate binding sequence (figure 4). Among various glycolytic enzymes phosphoglycerate mutase was found to be the best substrate for insulin-stimulated phosphorylation of tyrosine residues (20).   No ligand(s) in the interprotein contacts were found and each subunit has one domain. One mechanism of interglobular
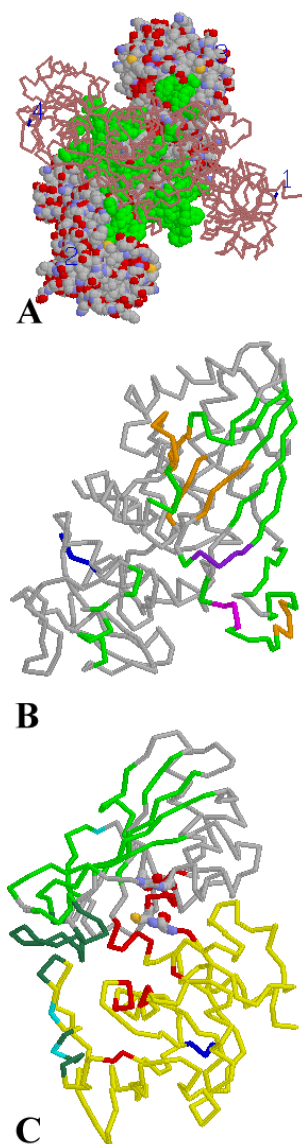
Figure 2. Glyceraldehyde-3-phosphate dehydrogenase from lobster. a) Tetramer of the enzyme (PDB: 4GPD). The molecule is formed by two crossing-over dimers, dimer 1-4 is shown using backbone representation, dimer 2-3 using spacefilling. NADp binding sequences (red) and interglobular contact regions (green) of the subunits 1-4 are shown. b) Subunit of the GAPDH with the functional sequence regions marked. N-terminal is marked with blue. Overlaps of interglobular contact sequences(green) with the PKC phosphorylation potential sites(magenta), CK2 sites (orange), CaM-2 sites (purple) are shown. c) Subunit of the GAPDH with the interglobular contacts and active site regions marked. N-terminal and C-terminal ("middle") domains of the subunit 1 are colored in yellow and gray respectively with the N-terminal marked (blue). Three interglobular contact regions IP12 (greenblue), IP13(cyan) and IP14(green) and active site signature/NADp binding sequences (red) are shown. Substrate phosphate binding site placed in a small cleft is shown in wire-frame mode (CPK coloring scheme).

contact- active site interaction was identified: sequence overlap (table 5). As gap between interglobular and ligand-binding sequences is 3-4, and hydrogen bonds within the monomer stabilize the binding loop, monomers may be partially active. This is confirmed by the work (3), where monomeric and dimeric PGM intermediates were found to possess partial activity (35% of the native enzyme).

4.5. Yeast enolase

Accessibilities of all kinase sites overlapping with interglobular sequences are changing upon dimerization, serines of the PKC and CaM2 sites become almost totally buried (table 6). Thus, modification of these sites may disrupt interglobular interaction. PKC phosphorylation (PKC 372-374) may also prevent or complicate substrate binding, CK2 phosphorylation (CK2 293-296) may induce destabilization of Mg- binding center (table 6). Despite the presence of overlaps between interglobular and substrate binding site sequences, first of them (159-161) involves His 159 that forms only one hydrogen bond with the bound substrate analog, and in the second one (374-379) the gap between sequences is relatively wide. At the same time, residues of stretch 6- 56 of N-terminal domain (1-128) and residues of stretch 161-417 of C-terminal domain(129-436) are involved in the interglobular interaction. Thus, main mechanism of enolase activation during oligomerization involves stabilization of interdomain interaction (figure 5). Particularly, interactions of cross-linking helix 402-417 may be important for this (table 7).

4.6. Summary of the results

Interglobular PKC and CK2 sites occur with the same frequency (4 enzymes from 5). Each of more rare tyrosine kinase and CaM2-kinase type sites occur near interglobular contact of two structures, other structures do not contain sites of these types. PKA sites are present in GAPDH and PGK, but none of them are near interglobular sequences (table 8). Percent of sites close to the region of interglobular contact is higher in tetramers than in dimers. Tetrameric GAPDH and PGM show unusually high percentage of kinase sites involvement in interglobular interactions. While in other enzymes only 15-25% of PKC, CK2 sites are involved, the tetramers have 40-50% of PKC sites in the interglobular contact, and GAPDH has 75% of CK2 sites in all the contacts. The figures for GAPDH are higher, as it has three interglobular contacts ("tetrahedron" tetramer), whereas PGM only two ("toroid" tetramer). In some of the enzymes (TIM, PGM) only sequence overlap mechanism was identified, whereas other enzymes, that contain two domains per subunit may have mixed mechanisms of activation with interdomain contact stabilization prevailing (table 8).

In the enzymes with two domains (GPA, ENL) the main activating oligomerization mechanism is interdomain contact stabilization, while in enzymes with subunits of one domain (TIM, PGM) the main mechanism seems to be functional sequence overlap. C-terminus domain contact stabilization in monomeric PGK may be considered as a kind of interdomain contact stabilization.

Table 4. Interdomain contact, kinase sites and ligand binding sequences in the structure of yeast phosphoglycerate kinase (PDB: 3PGK). There are 8 PKC, 6 CK2 and 1 CaM2 in the sequence. Interdomain contact was analyzed on the base of bond list for the whole molecule

Sequence type	N-term. domain	C-term. Domain
	1-197,(402-415)	198-401
Interdomain contact	163; 189-194	365-367; 388-390; 395-400
C-terminal	5-10; 165-168	(405-415)
PKC sites	3-5 (OG SER 1.3)	(412-414), (OG Ser 7.5)
CK2 site	7-10(OG SER 0.0)	
CaM2 site	168-171	
ATP-binding		211-213; 289; 311; 334-338
MG-binding		ATP; Asp 372
Substrate binding		392-393 HB/HP
'Basic' patch	62-65, 167-170	

Table 5. Overlaps of interdomain contact, kinase sites and active site sequences in yeast phosphoglycerate mutase (PDB: 3PGM, 1BQ3; 8 PKC, 1 CK2, and 3 TyrK sites). For monomer and tetramer molecules absolute accessibility of hydroxyl atom and relative residue solvent accessibility are given.

Sequence 1	Sequence 2	Accessibility data for Ser/Tyr residues	
		Monomer	Tetramer
IPAC 82 83	PKC 81 83	0.3, 0.03	0.0, 0.0
IPAC 168 171	PKC 172 174	5.4, 0.16	5.4, 0.14
IPAB 26 26	PKC 28 30	0.1, 0.09	0.0,0.06
IPAB 69 80	PKC 81 83	---/---	---/---
IPAC 82 83	TyrK 83 89	0.1, 0.11	0.0, 0.04
IPAC 139 144	TyrK 132 139	3.5, 0.21	3.5, 0.17
IPAB 135 136	TyrK 132 139		
		Comments	
IPAC 82 83	MP31 86 86	Glu 86, HP contacts with ligand	
IPAB 61 65	ACTs 59 59	Arg 59, no contacts found.	
PKC 184 186	ACTs 181 181		
PKC 172 174	MP31 179 179		
TyrK 83 89	MP31 86 86		

Table 6. Overlaps of kinase site, interglobular and ligand binding sequences in the structure of yeast enolase (PDB: 1EBG). Maximal overlap gap is 5, there are 6 PKC, 6 CK2, 2 CaM2 sites in the sequence. Solvent accessibility values are given as OG Ser atom accessibilities and relative accessibilities for whole Ser residues in monomer and dimer.

Sequence 1	Sequence 2	accessibilities, comments			
		Monomer		Dimer	
		OG	Res.	OG	Res.
IPAB 402 417	PKC 403 405	8.0	0.22	0.5	0.01
IPAB 6 15	CK2 9 12	1.9	0.13	1.9	0.12
IPAB 183 184	CaM2 184 187	4.3	0.13	0.0	0.04
IPAB 161 161	PAH1 159 159				
IPAB 379 379	PAH1 374 375				
PKC 372 374	PAH1 374 375	Prevention of substrate binding			
CK2 293 296	MG1 295 295	Destabilization of Mg coordination			

Table 7. Cross-linkage of the enolase domains of each subunit by interactions with helix 403-419 of the neighboring one (only hydrogen bonds are listed, SSE- secondary structure element).

Residue 1	Residue 2	Atom 1	Atom 2	SSE 1	SSE 2
TYR 6 A	GLU 417 B	OH	OE1	STRAND 4 12	HELIX 403 419
TYR 6 A	GLU 417 B	OH	OE2	STRAND 4 12	HELIX 403 419
ARG 8 A	GLU 417 B	NE	OE2	STRAND 4 12	HELIX 403 419
ARG 8 A	GLU 417 B	NH2	OE1	STRAND 4 12	HELIX 403 419
GLU 20 A	ARG 414 B	OE1	NH2	STRAND 16 25	HELIX 403 419
GLU 20 A	ARG 414 B	OE2	NE	STRAND 16 25	HELIX 403 419
GLU 379 A	ARG 414 B	OE2	NH2	LOOP 373 382	HELIX 403 419
GLU 379 A	ASN 410 B	OE2	ND2	LOOP 373 382	HELIX 403 419
SER 403 A	GLU 404 B	N	OE1	LOOP 396 403	HELIX 403 419

Activating oligomerization
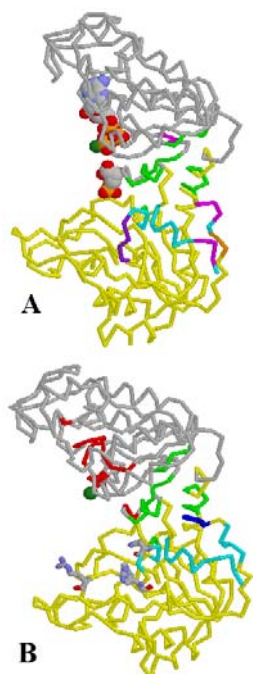
Figure 3. Yeast phosphoglycerate kinase. a) Interdomain contacting sequences and kinase sites. C-alpha trace of the monomeric enzyme and bound molecules are shown. N- and C-terminal domains are colored in yellow and gray. Overlaps of kinase sites involved both in the interdomain contact and interaction of the C-terminal are shown (PKC- magenta; CK2-orange; CAM2- purple) along with Interdomain sequences (green) and C-terminal interacting sequence (cyan). b) Interdomain sequences and active site/ binding site regions. Mg-ion (dark green spot) is bound by ATP phosphate atoms (not shown) and by Asp 372. ATP-binding, phosphoglycerate-binding sequences and Asp 372 involved in the Mg-binding are shown in red, 'basic' patch residues (N-domain: His 62, Arg 65, His 167 and His 170) are shown using wireframe (CPK coloring).
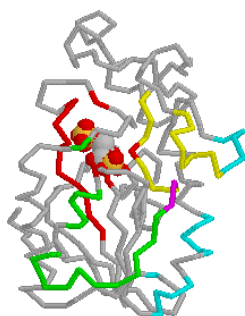
Figure 4. Yeast phosphoglycerate mutase. One one-domain subunit of the "toroid" homotetrameric molecule (two interglobular contacts in tetramer) with bound phosphoglycerate molecule and functional sequences marked is shown. Both interglobular contacts AB (dimer, green), AC (cyan) are of equal size. Overlaps of interglobular contact sequences with PKC site (magenta), tyrosine kinase sites (yellow) and active site sequences (PGM signature and phosphoglycerate binding site, red color) are shown.

In all the structures investigated N- and C-terminal fragments are interact one with another in each subunit, some of chain termini are involved in interglobular interactions (table 9). Interactions of chain termini are important as the terminals may be easily unfolded and modified. Termini (or terminals) of polypeptide chains are also highly susceptible to proteolytic digestion and thus states of N,C-terminals may also define half time of enzyme's life. Terminals often contain post-translational sites (for example, PKC 3-5 and CK2 7-10 in PGK, table 4). Exhaustive statistical analysis of the amino acid sequences at the carboxyl-terminals of proteins has shown (*E. coli*, Yeast, and Homo Sapiens sequences) that positively charged amino acid residues are over-represented while Gly residues are under- represented (21). Thus, despite that C-terminals are forming one geometrical unit with N-domains in two-domain proteins (GAPDH, PGK, and ENL), their interactions are to be considered separately in structural and biochemical analyses. In average, number of most frequent PKC and CK2 sites placed both in interglobular contact region and on active/binding sites is in 30-60% range. The possible roles of remaining sites are considered in the "Discussion" section.

5. DISCUSSION

5.1. Algorithm of protein-protein contacts analysis

In the most programs for protein-protein interaction analysis for identification of the interacting atoms involves usage of distance cutoffs. However, as these cutoffs are usually in somewhat wide range of 7..12 angstrom and as their usage implies complete ignoring of basic principles of protein physical chemistry, in the present work interactions between neighboring globules were analyzed in terms of hydrogen bonds and hydrophobic interactions.

5.1.1. Criteria of hydrogen bond identification

Hydrogen bond is an interaction between two electronegative atoms, donor and acceptor, through an intermediate atom of hydrogen covalently connected with the donor (22). As resolution of most of XRay data lies hydrogen atom positions could not be defined on the base of electronic density map and calculation of the positions on the base of known coordinates of donors and their geometries is necessary. In this work a simple method of hydrogen atoms calculations (for atoms- hydrogen donors) based on similarity of configurations of hydrogen donating atoms was used. For sp3-donors ("rotating hydrogen") possible coordinates of hydrogen atoms were analyzed during identification of hydrogen bonding interactions. As hydrogen bonds are considered as a kind of ion interaction (22), the bonds are to be characterized using intervals of values. For the definition of these intervalsspecific in the case of proteins it is necessary to carry out measurements of these parameters on a set of protein structures. Recent results were presented in the papers (22,23). The criteria proposed in the work (23) were analyzed from geometrical viewpoint and minimal set of geometric criteria for hydrogen bond identification was proposed (unpublished data). The hydrogen bond is identified if distance hydrogen- acceptor is less than

Table 8. Kinase sites in interglobular contacts of the glycolytic enzymes studied and mechanisms of interglobular contact/active site interaction

Enzyme	NDom	Oligomer	PKC		CK2		TyrK		CaM2		Mechanism
			IPC	AS	IPC	AS	IPC	AS	IPC	AS	
TIM	1	Dimer	1/5	2/5	1/6	-	-	-	-	-	SQO
GAPDH	2	Tetrahedron tetramer	2/4	1/4	6/8	2/8	1/1	-	1/1	-	ICS,(?ILB)
PGK	2	Monomer	2/8	2/8	1/6	-	-	-	-	-	ICS, SQO
PGM	1	Toroid tetramer	3/8	2/8	-	-	2/3	1/3	-	-	SQO
ENL	2	dimer	1/6	1/6	1/6	1/6	-	-	1/2	-	ICS, (SQO)

(Abbreviations: NDom- number of domains, IPC- interprotein contact, AS- active site, SQO- sequence overlap, ICS-interdomain contact stabilization, ILB- interglobular ligand binding (a potential mechanism for GAPDH)).

Table 9. Intraglobular and interglobular interactions of chain termini in the proteins studied

Intraglobular interactions				
Enzyme	SSE(s) 1	SSE(s) 2	HB	HP
TIM	loop, strand 5 11	Helix 239 246	0	1
GAPDH	helix 13 25	Helix 317 333	0	5
PGK	loop 1 17	Loop 410 414	1	20
PGM	loop 1 3	Loop 211 224	1	1
ENL	loop 12 16	Helix 403 419	0	3
Interglobular interactions				
TIM	loop 11 16	loop 11 16	5	6
ENL	strand 4 12	helix 403 419, helix 179 200	6	7

HB- number of hydrogen bonds, HP- number of hydrophobic contacts.
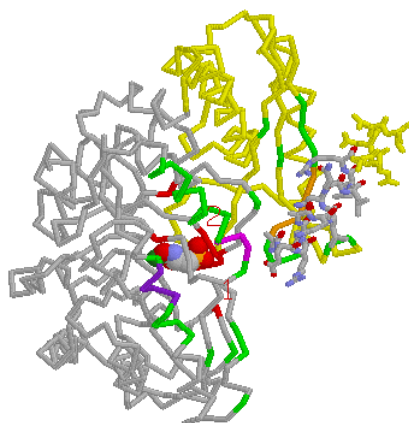
Figure 5. "A" globule of the yeast enolase dimer. N-domain is marked in yellow. Parts of the helix 403-419 of the B globule cross-linking domains in the dimer contacting with domains of the A globule are colored in accordance with the domain contacted (wire-frame). Interglobular contact sequences (green), PKC, CK2, CaM2 sites in the interglobular contact (magenta, orange, purple), two Mg-ions and bound substrate analog molecule (space-filling) are shown. Two overlaps with ligand binding sequences (red) are marked (1: 159-161; 2:374-379).

sum of the Van der Waals radii of the two atoms (max. 2.5 angstrom) and angle donor-hydrogen-acceptor is higher than 90.0 degrees. Instead of checking values of the AA-A-H and AA-A-D angles, proposed in (23) as an additional criterion, unfavorable main-chain interactions between O atom of each (i) and N atom of each (i+2) residue were simply excluded from analysis.

5.1.2. Criterion of hydrophobic interaction

Hydrophobic interaction is defined as a cluster of non-polar groups given water removed from the volume of the cluster (24). This definition could not be used for automatic structural calculations. Thus, in this work simplest distant criterion was used: two hydrophobic atoms are considered to be in atomic hydrophobic interaction, if distance between them less or equal to the sum of their Van der Waals radii. The definition is somewhat wide, as using it amino acid with completely non-polar side chains (Val, Ile...), but also having polar ones (Arg, Lys, Glu...). would be identified as participating in hydrophobic interactions. In addition, using only this criterion ignores the fact that hydrophobic interaction is a group one. However, as the primary task is to find functional sequences of residues, this criterion is appropriate.

5.1.3. Identification of amino acids involved in intra-protein and inter-protein interactions and ligand binding sites

Each amino acid may be represented as pair of spheres. Inside one of them (B-sphere) are atoms forming peptide bonds (N,CA,C,O), inside the other (S-sphere)- atoms of side chain. Coordinates of each sphere center are calculated as mass center and the radius as distance from the center of the most distant atom plus it's Van der Waals radius. For amino acids having side-chains with no more than one atom (GLY, ALA), the atoms CA (C-alpha) and CB (C-beta) are chosen as S-spheres. As the method of sphere size calculation gives maximal radius of amino acid, this is sufficient for finding all amino acids, placed in the region of inter-protein contact. Any two spheres of given two residues are in contact, if distance between sphere

centers is less than sum of radii. This condition allows selecting amino acids placed near surfaces of inter-protein contact or contacting each other inside one globule of the protein. For finding amino acids participating in interaction non-covalent interactions (hydrogen bonds and atomic hydrophobic interactions) between atoms of the two contacting amino acid are checked.

General scheme of the algorithm is as follows: there are two globules, each is presented as a folded chain of spheres (like beads). Residues of one globule are consecutively checked for overlaps of its spheres with any residues of the globule. If there is an overlap between two spheres then presence of hydrogen bonds and hydrophobic interactions is checked. The obtained bond list for all the residues is used then for extraction of the functional sequences.

The most reliable way to define active site (ligand-binding) groups in an enzyme is to use available data on enzyme-substrate complexes, prepared using substrate analogue or under conditions, when enzyme is inactive. The described algorithm may be also used for identification of interactions inside one globule and it was used for finding ligand-binding sequences. In this work were used PDB entries that contain coordinates of the bound ligands. The same procedure allows finding atoms and residues interacting with the ligands.

5.2. Oligomerization, activity and conformational change

The phenomenon of activating oligomerization, analyzed here on the example of pay-off phase glycolytic enzymes, implies that monomers are inactive (or less active/have other functions) while oligomers are active. However, this does not represent all possible relations between oligomeric states of a protein and it's activity. For example, protein kinase A is active in monomeric state, while inactive in the dimeric (25). Catalytic activity of phosphorylase kinase is associated only with the gamma subunit, while being inhibited by addition of alpha/beta subunits (26). The two enzymes are heterodimers. Homodimeric glycogen phosphorylase is inactive as monomer, active as dimer and inactive in tetrameric form (27).

Protein kinase A, as well as a number of other proteins of signal transduction undergoes significant conformation changes upon dynamic dimerization (28), while it is not the case of the stable (static) dimers/oligomers, considered in this article. Differences between dynamic and stable-enzyme oligomerization are mainly in significant conformational changes that are characteristic for many dynamic interactions (28). Signal transduction events are also considered further in this article.

Dynamic conformation transitions of a protein in solution may be controlled using some spectroscopic methods (as light scattering and fluorescence measurements (29)) or NMR methods in the most cases. However, the latter is applicable only to relatively small proteins, while widely used methods of the former group give rather general information on protein conformation. Possibly due to this the concept of "conformational change" has evolved. This concept represents biochemical viewpoint, when a little or almost no structural information is available. The change implies some change in the general globule conformation that leads to change in properties. In general case, no "rules of a thumb" could be proposed for studying the influence of this change on activity. Moreover, often the active site is affected before noticeable conformational changes can be detected for the enzyme molecule as a whole (29).

Structural mechanisms of activating oligomerization, established in this work allow making "conformational change" concept somewhat concrete. If a local change in conformation (helix shift or loop unfolding, for example) occurs during oligomerization it could hardly be registered by the widespread methods. The domain association/partial dissociation also may lead to a small change in the spectra, as domains represent relatively independent, 'hard' units. The same concerns ligand-induced subunit association. Thus, the elaborated analysis of XRay-based "static" protein spatial structure allows to distinct between at least three types of "invisible" conformational change, that corresponds to domain/subunit association in the case of oligomeric or multidomain proteins.

5.3. Potential cellular functions of enzyme oligomerization

Oligomerization is important for enzyme activation/inactivation in metabolic pathways, regulation of stability (and therefore, lifetime) of enzymes. Particularly, it provides protection from degradation: the monomer of phosphoglycerate mutase with 35% of full activity is sensitive to trypsin, while dimer and tetramer are not (3). Changes in oligomeric state not only affect activity of the enzymes, but also may drastically affect their cellular location and functions. This is illustrated below on the example of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Sequence of nuclear protein uracil DNA glycosylase (UDG) was found to be completely homologous with the 37-kDa subunit of human GAPDH. A 37-KDA subunit (i.e. GAPDH monomer) was shown to possess UDG activity equivalent to that seen for the purified human placental UDG (30). Two high-affinity cAMP-binding proteins (I and II) from baker's yeast have been identified as different oligomeric forms of GAPDH (31). Oligomerization of GAPDH may have even more important function. Analysis of subcellular enzyme distribution has showed possibility that neuronal apoptosis may be triggered by GAPDH accumulation in the nucleus, resulting in perturbation of nuclear function and ultimate cell death (32). As it was mentioned above, there are evidences that nuclear GAPDH is monomeric (30,33).

GAPDH is not the only glycolytic protein that was found in nucleus in modified form. Numerous studies have demonstrated the presence of at least four glycolytic enzymes in the nuclear compartment of several cell systems (34): lactate dehydrogenase, phosphoglycerate kinase, and aldolase and glyceraldehyde-3-phosphate dehydrogenase.

Activating oligomerization

All four proteins have been reported to bind DNA and in some cases the nuclear enzymes were found to be a modified form of the cytoplasmic. Their association with DNA was supposed to have a role in DNA transcription/replication through non-specific stabilization of chromatin structure (34).

5.4. Cellular regulation of protein-protein association

Protein-protein association is strongly dependent on pH, ionic strength and temperature. However, these influences are non-specific. Only certain values of intracellular pH and only narrow ranges of inorganic ion concentrations optimal for life are found in the cytoplasm of the cells of most animals, plants, and microorganisms. This is necessary for maintaining adequate rates of catalysis, high level of regulatory responsiveness and other characteristics of the whole cell medium (35). Although, changes in pH particularly affect glycolysis: at more alkaline pH, glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase reactions may become the rate-limiting steps of glycolysis (36).

Ligand-induced dimerization (oligomerization), also termed in this work as "interglobular ligand binding" is more specific way of affecting oligomeric states of enzymes. Here already mentioned ATP-mediated oligomerization of phosphofructokinase may be referred to. In some cases ligand/coenzyme molecule may stabilize the oligomeric form: e.g., in the absence of NAD+ the GAPDH dimer inactivates irreversibly (37). The extent of dimerization of citrate synthase was found to be dependent on the concentration of the enzyme, pH, ionic strength and concentrations of oxaloacetate and citrate, substrates for the forward and reverse reactions respectively (38). Depending on type of allosteric activators present in the media some enzymes may have multiple oligomeric forms. Thus, four distinct types of pyruvate decarboxylase tetramers have been observed. Addition of definite ligands induces drastic changes in the mode of oligomer assembly (39).

Protein-protein association is also subject to regulation through kinase phosphorylation. Binding of GAPDH and aldolase to the N-terminus of human erythrocyte anion transporter, band 3, is disturbed upon tyrosine kinase phosphorylation of band 3. In this case tyrosine phosphorylation prevents protein-protein association, in contrast to the case of SH2 domains where phosphorylation is required for binding (40).

5.5. Regulated oligomerization and signal transduction

Regulated dynamic protein dimerization (oligomerization) is involved in many transduction pathways. Specific protein-protein interactions are essential for almost all biological processes. Dynamic protein-protein interactions are a key component of biological regulatory networks, they are frequently employed in signal transduction from the cell surface to the nucleus (28). Static protein-protein interactions as interactions between subunits of an oligomeric enzyme which are the subject of the present paper may represent another type of regulated oligomerization.

Most of signal transduction pathways go through second messengers and then related protein kinase cascades. cAMP-dependent phosphorylation performed by PKA involves several separate stages: binding of signal molecule to the receptor, G-proteins, adenylate cyclase, PKA. Calcium-dependent phosphorylation involves: receptor, phospholypase/diacylglycerol/calcium, PKC. These stages represent "general" stages of signal transduction and involve dynamic oligomerization. Present work shows possibility that "specific" transduction events involving kinase phosphorylation of multiple cellular proteins (terminal signaling events) may involve regulation of "static" protein-protein interactions. In this case, immediate downstream targets of, as example, PKC may be not only other kinases specific for terminal proteins (as phosphorylase kinase) but also the terminal proteins themselves may be directly regulated by "common type" kinases.

Metabolic regulation of gene expression leads to changes in the *number of the synthesized* protein molecules. Regulation of oligomer formation influences the number of *active* oligomeric molecules thus having significant impact on the enzyme's total activity in cell in "all-or- nothing" mode (as monomers are inactive and mostly oligomers are significantly active).

5.6. Influence of kinase phosphorylation on formation of stable oligomers and putative structural mechanisms of kinase phosphorylation

Kinase regulation through activating oligomerization becomes important when enzymes may be substrates for corresponding kinases. Epidermal growth factor (EGF) receptor tyrosine kinase phosphorylates PGM and GAPDH *in vitro* (41). Both enzymes contain tyrosine kinase sites (table 8). Rabbit muscle enolase was found to be phosphorylated *in vitro* by PKC (42). GAPDH is also directly phosphorylated by protein kinase C, involved in the regulation of cellular motility and energy metabolism (43). Although some of phosphorylation results are for enzymes from other eukaryotic sources, nevertheless this shows possibility of modification.

In spite of that kinase sites are presented in many sequences, detailed data on phosphorylation are not available for any enzyme under interest and mostly indirect biochemical evidences may be found. Even if there was some research made on phosphorylation/mutagenesis of some particular sites in some proteins, the results are rarely complete. In the case of phosphorylated peptide analysis, phosphoresidues are easily hydrolyzed, thus a large portion of phosphorylated residues could not be identified. Only few works are dealing with comparably non-destructive analysis of protein phosphorylation: "fixation" of the modified residue (44) or usage of non-protein solvent (acetonitril) during chromatography of the digested peptides (45). These methods are not of wide use. In the case of kinase site mutagenesis, there is significant number of potential sites to be mutated/deleted and the works of this kind are also rare.

In this work potential value of some kinase sites on the base of protein spatial structure and sequence site data

was assessed. Kinase sites placed in interglobular contact regions may be not less important than those placed on the ligand binding/active site sequence (table 8). Moreover, due to 'hardness' of interglobular contact regions (see below) the interglobular sites in monomer may be subject to multiple phosphorylation/dephosphorylation cycles, whereas modification of ligand binding sequences may lead to irreversible unfolding of the active site.

For kinase regulation of enzymatic activity through oligomerization to be possible, two principal structural conditions must be observed: 1. kinase sites are to be in the interglobular contact region(s); 2. there is to be definite influence of interglobular contact on activity. The second moment is also supported by the biochemical data on oligomer/monomer activity.

Why could one state that phosphorylation of interglobular contact region of a subunit prior to its association into dimer would definitely influence macromolecular assembly ? Most of interglobular contact regions are planar and the planarity is most possibly not induced due to protein-protein association (46). Addition of phosphate (a molecule of 5-7 angstrom in size) leads to appearance of a knob without complementary cavity on another subunit. As conformational changes upon the association are insignificant, in other words, individual subunits are 'hard', the accommodation of the attached phosphate due to distortion of other subunit is hardly probable. Therefore, if this modification does not prevent oligomer formation completely, at least it significantly lowers stability of oligomer, thus diminishing it's half-life in the cell. In this article, surface accessibility values were used for assessing possible structural consequences of a site's phosphorylation. If values for a sequence in monomer and dimer differ significantly and accessibility of the hydroxyl group in dimer(oligomer) is close to zero, modification the site may cause significant disruption of the oligomer structure. Otherwise, it could not be defined whether site's modification lead to disruption or misformation or to some stabilization of the oligomer.

Definite structural evidences of interglobular contact- active site connection in oligomeric enzymes studied in this work points to the stabilization of the active conformation due to interglobular interaction. Therefore, dissociation of the oligomer would inevitably lead to significant distortion of the active site conformation due to partial unfolding of this important functional region and loss or significant loss in catalytic activity.

On the base of the activating oligomerization mechanisms established in the work, the following structural mechanisms of kinase phosphorylation influence may be proposed: 1. *GLOBAL CONFORMATIONAL CHANGE*: phosphorylation may lead to a complex change in conformation of the modified molecule due to sidechain interactions with the attached phosphate (47). Obtaining any definite results requires complex procedure of molecular modeling of the molecule with attached phosphate. 2. *LOCAL CONFORMATIONAL CHANGE*: phosphorylation of sites consecutive to active sites regions.

Although percentage of these sites is somewhat lower, that of the sites in the interglobular regions (table 8), modification even of one site may completely abolish enzyme's activity due to prevention of substrate binding, modification of important catalytic residues or due to distortion of active site conformation. Phosphorylation may also modify the mode of regulation through allosteric metabolites by preventing allosteric ligand binding. 3. *INTERGLOBULAR CONTACT MODIFICATION*: three mechanisms were already presented. This type of regulation is possible only for oligomeric and/or multidomain proteins. As in assembled functional macromolecules the interglobular phosphorylation sites are protected, this type of regulation is possible when monomers are available in cell.

5.7. Implications for metabolic analysis

Analysis based on spatial structures of a functional group of enzymes and corresponding sets of biochemical data - if available may allow to assess impact of some or other signal pathway not only on a concrete protein but also on a protein functional group. However, several problems consternate such an analysis:

1. Each enzyme may presented in several metabolic pathways, which may have different cellular location and therefore, kinase accesibility. 2. For assessing kinase influence, it is necessary to find function/influence of each kinase site for each enzyme. Three mechanisms of kinase phosphorylation proposed above may help in doing this. The remaining problems are possible presence of non-consensus phosphorylation and that not all functional regions of an enzyme may be known. 3. Complex character of influence (not simply "activation/inactivation"). For example, PKC phosphorylation of enolase results in activation of the forward reaction and inhibition of the backward one (42). Nevertheless, some helpful conclusions may be drawn out on the base of data such as summarized in the table 8.

A. CK2 and PKC kinases are important for cell division processes. Casein kinase 2 (CK2) is required for cell's viability and for cell cycle progression. It's activity is independent on intracellular concentrations of cyclic nucleotides and calcium (48). PKC kinases are involved in mitogenic signal transduction, involved in remodeling of the actin cytoskeleton as well as apoptosis (49).

Cell cycle processes involve significant rearrangement of DNA. Monomeric glycolytic enzymes may function as non-specific DNA binding proteins stabilizing DNA structure, as it was already mentioned. As both kinases are inevitable for the cell cycle, one of their functions (at least for the studied glycolytic enzymes) may be shifting of monomer/oligomer equilibrium in the direction of monomers, which are important for DNA stabilization. Involvement of PKC in apoptosis, that involves global DNA rearrangements (49) corresponds with higher percent of PKC site interglobular sequence occurrences in comparison to other site types (table 8).

B. In addition to presence of an enzyme in several metabolic pathways, principal difference of *in vitro*

and *in vivo* media is that not all protein-protein interactions are conserved *in vitro*. Absence of any effect *in vitro* upon kinase site modification/deletion does not necessarily imply that site is non-functional *in vivo*.

For most of the proteins studied no more than 50% of PKC and CK2 sites are involved both in interglobular contacts and active site sequences. One of the most important roles of the remaining sites may be regulation of association with other proteins. Pairwise interactions between glycolytic enzymes and interactions with cytoskeleton are of primary importance. Recent studies have demonstrated that most glycolytic enzymes can reversibly associate to form heterogeneous enzyme-enzyme (binary) complexes *in vitro*. Individual enzymes have a varied response to complex formation: some enzymes are inhibited, some are activated and some are unaffected (50). Interactions of consequtive glycolytic enzymes may be important for substrate chanelling. 1,3-diphosphoglycerate may be directly transferred between GAPDH and PGK via an enzyme-substrate-enzyme complex (51). The interaction of rabbit skeletal muscle enolase and PGM was also detected (52).

Interactions of the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase, triose-phosphate isomerase, enolase, phosphoglycerate mutase, phosphoglycerate kinase with cytoskeletal components, such as microtubules, may play structural role in the formation of the microtrabecular lattice (53). Glyceraldehyde 3-phosphate dehydrogenase interacts with *in vitro* reconstituted microtubules which leads to partial inhibition of the activity of the microtubule-bound enzyme (2). At the same time F-actin forms a complex with the enzyme and increases it's activity (54). Phosphorylation of kinase sites, which are mostly placed on surfaces of the proteins studied, may be crucial in regulation of all the mentioned types of cellular protein-protein associations.

6. PERSPECTIVE

Activating oligomerization, or acquiring (full) enzymatic activity upon oligomer formation, represents a simplest form of biomolecular collective behavior. Oligomerization is also important for protein stability and maintaining specific range of activities.

Proposed in this work procedure of sequence and spatial structure based analysis allows to establish mechanisms of activating oligomerization and possible ways of regulating oligomerization through kinases. In some of the enzymes only sequence overlap mechanism was identified, whereas other enzymes, that contain two domains per subunit may have mixed mechanisms with interdomain contact stabilization mechanism prevailing. Structural mechanisms of activating oligomerization allow to pinpoint key residues and thus to give somewhat detailed picture of the process on molecular level. Interactions of polypeptide chain termini may be important both for protein stabilization and kinase regulation.

Biological roles of inactive monomer- active oligomer and vice versa transition are discussed. Changes in oligomeric state not only affect activity of the enzymes, but also affect protein cellular location and functions. Activating oligomerization is supposed to be regulated mainly through signal transduction pathways on the level of kinase phosphorylation. Regulation of oligomer formation influences the number of active oligomeric molecules and thus may have significant impact on the total cellular activity of the oligomeric enzymes. As no more than 50% of PKC and CK2 sites are involved both in interglobular contacts and active site sequences, one of the most important roles of the remaining sites may be regulation of association with other cellular proteins.

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