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Biophysical characterization of Gir2, a highly acidic protein of *Saccharomyces cerevisiae* with anomalous electrophoretic behavior☆

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Abstract

Gir2 is an uncharacterized protein of *Saccharomyces cerevisiae*, containing a RWD/GI domain. In this work, we report the biophysical characterization of Gir2. His-tagged Gir2, expressed and purified from *Escherichia coli*, showed an abnormally slow migration on SDS–PAGE. The yeast expressed protein behaves similarly. Using mass spectrometry and peptide mass fingerprinting we demonstrated that the protein has the expected molecular mass (34 kDa). EDC modification of carboxylate groups reverted the anomalous migration on SDS–PAGE. Size exclusion chromatography showed that Gir2 has a Stokes radius larger than expected. Gir2 is thermostable and lacks extensive structure, as determined by CD analysis. Based on these findings, we suggest that Gir2 is a representative of the growing group of "natively unfolded" proteins.

Keywords: Gir2; Highly acidic; Anomalous behavior; Natively unfolded

Gir2 (YDR152W) (GenBank Accession No. NP 010436.1) is an as yet uncharacterized protein of *Saccharomyces cerevisiae* which contains a RWD domain (domain in RING finger and WD repeat containing proteins, and in DEXDc-like helicase subfamily related to the UBCc domain) at its N-terminus [1]. This domain is also found in Gcn2, a kinase that phosphorylates eIF2, and in Yih1 and its mammalian ortholog, Impact, and is called GI domain. The GIR2 gene is non-essential and its deletion does not impart any apparent phenotype to yeast cells (http://www.yeastgenome.org). Gir2 has high similarity with human hypothetical protein PTD013 and *Caenorhabditis elegans* hypothetical protein T26E3.4.p (GenBank Accessions Nos. NP_057036.1 and NP_493207.1, respectively).

Gir2 has a predicted molecular mass of 31 kDa with a high content of acidic amino acids (23.5%), giving a high negative net charge (−30) at pH 7.0. The analysis of the primary sequence predicts a potential N-terminal PEST sequence, which targets proteins for rapid degradation [2].

In order to obtain structural and functional information about this protein we expressed and purified a His-tagged recombinant form of Gir2 in *Escherichia coli*. The recombinant protein showed an uncommon slow migration in SDS–PAGE, also seen in the yeast expressed protein. Therefore, we studied the recombinant Gir2 by mass spectrometry, size exclusion chromatography, chemical modification of acidic amino acids, and circular dichroism analysis. Using these approaches, we demonstrate that the anomalous migration of Gir2 in SDS–PAGE is due to its high acidic amino acid content and suggest a natively unfolded structure for this protein.

Materials and methods

Expression and purification of His6-Gir2. The coding sequence of Gir2 from the yeast *S. cerevisisae* was obtained by PCR from genomic

☆ *Abbreviations:* MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; Q-TOF, quadrupole time-of-flight; PMF, peptide mass fingerprinting; EDC, 1-ethyl-(3-(3-dimethylamino)propyl) carbodiimide; CD, circular dichroism; DLS, dynamic light scattering; BSA, bovine serum albumin.

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DNA using the oligonucleotides BC218F (5′-GGGGGATCCATGG ATTATAAGG AAGAACAGAAGCAGGAACTA-3′) and BC218R (5′-GGGGGGGCCCTTATTGATTTGCTATTTCGGTCTTGGCA AGTC-3′). The PCR product was ligated into pUC18 *Sma*I/BAP, using the Sure Clone Ligation Kit (Invitrogen), creating plasmid pBE427. The insert was throughly sequenced. For expression of the complete protein (amino acids 1–265), the *Bam*HI–*Pvu*II fragment of pBE427 was ligated into the *Bam*HI–*Hin*dIII sites of pET28-a(+), with the *Hin*dIII terminus made blunt by treatment with the Klenow fragment of DNA polymerase, creating plasmid pBE430. To express a truncated form of Gir2 (amino acids 1–166), the *Bam*HI–*Xho*I fragment of pBE427 was cloned in the *Bam*HI–*Xho*I sites of pET28-a(+), creating plasmid pBE433. In both constructions, the expressed proteins should have 33 extra amino acid residues added to the N-terminal end, derived from the vector sequences, excluding the initial methionine residue which should be processed. His-tagged Gir2 was expressed in *Escherichia coli* BL21(DE3) at 23 °C for 18 h by adding 0.1 mM IPTG and purified using NiNTA-resin (Qiagen). Bacterial cell lysates containing His6-Gir2$^{[1-265]}$ or His6-Gir2$^{[1-166]}$ were incubated with nickel-chelating resin in lysis buffer (PBS, 10 mM imidazole) at 4 °C for 1 h and then washed with buffers (300 mM NaCl, 50 mM Na_2HPO_4, pH 8.0) containing 10 and 30 mM imidazole. The recombinant proteins were eluted off the resin with the same wash buffer containing 250 mM imidazole. Purified protein was dialyzed against 20 mM Tris–HCl, pH 8.0.

Expression of Gir2 in S. cerevisiae. The expression of native Gir2 was analyzed in strain BY4741 (*MATa*, *his3*Δ1, *leu2*Δ0, *ura3*Δ0, *met15*Δ0), using as control the isogenic strain #3510 (*MATa*, *his3*Δ1, *leu2*Δ0, *ura3*Δ0, *met15*Δ0, ydr152w::*kan*MX4) (Resgen) deleted of the chromosomal copy of GIR2. For overexpression of the complete protein in *S. cerevisiae* the *Bam*HI–*Pvu*II fragment of pBE427 was ligated into the *Bam*HI–*Hin*dIII sites of the pGAL1-10 plasmid pBM272 (*URA*3, *CEN*4, and *ARS*1) [3], after filling in the *Hin*dIII terminus with the Klenow fragment of DNA polymerase, creating plasmid pBE486. Yeast strain H1402 (*MAT*α, *ura3*-52, *leu2*-3, -112, *ino1*, *GCN2*, <*HIS4-lacZ*, *ura3*-52>@ *ura3*-52) was transformed with pBE486 and Gir2 was overexpressed, under the control of the GAL1 promoter, at 30 °C by adding 10% galactose to logarithmic growing cells in S minimal medium supplemented with 2% raffinose and required amino acids. Standard yeast genetic techniques and media were used as described elsewhere [4].

Immunoblots. Yeast whole cell extract was prepared in buffer containing 20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 30 mM $MgCl_2$, and 1 mM PMSF and subjected to SDS–PAGE. The proteins were blotted to a Hybond-C membrane (Amersham) and probed with mouse antiserum raised against purified His6-Gir2, followed by incubation with protein A–horseradish peroxidase (1:4000, Sigma) and detection using ECL detection kit (Amersham). Mouse antiserum was produced by intra-peritoneal injection of 100 μg purified protein with complete Freund's adjuvant (Sigma) followed 15 days later by a second administration of 100 μg protein in incomplete Freund's adjuvant. Blood was collected 30 days after the first immunization. For antiserum preparation the blood was incubated for 30 min at 37 °C, followed by 18 h at 4 °C and centrifuged at 500*g* during 15 min.

Molecular mass determination by size exclusion chromatography. Gel filtration chromatography was carried out using a Superdex 200 HR 10/30 column (Amersham Biosciences) with a flow rate of 0.3 ml/min at room temperature in 20 mM Tris–HCl, pH 8.0, containing 150 mM NaCl with a FPLC (Pharmacia). The column was calibrated with BSA (67 kDa) and lysozyme (14.3 kDa). The distribution coefficients (K_{av}) of His-tagged Gir2 were compared to the standard proteins to determine the M_r value. Results from gel filtration were used to calculate the Stokes radius (R_s) using the empirical equation [$\log(R_s) = 0.369 \log(M_r) - 0.254$] [5].

Molecular mass determination by MALDI-TOF spectrometry. Samples of purified His6-Gir2$^{[1-265]}$ and His6-Gir2$^{[1-166]}$ at 2 mg/ml in 20 mM Tris–HCl, pH 8.0, were dialyzed against water. The precipitated proteins were centrifuged at 10,000*g* for 15 min and solubilized in 0.1% formic acid. Samples of 2 μl were applied to a MALDI-TOF/Pro mass spectrometer (Amersham Biosciences) with a matrix of sinapinic acid. The mass was assigned with the automatic mode of acquisition of the spectrometer control software.

Peptide mass fingerprinting. Samples of purified His6-Gir2$^{[1-265]}$ at 2 mg/ml were solubilized in 0.1% formic acid, diluted in 50 mM bicarbonate buffer, and digested with 2 nM trypsin at 30 °C for 1 h. The protease digested mixture was mixed with a matrix of α-cyano-4-hydroxycinnamic acid and applied to MALDI-TOF/Pro mass spectrometer in reflectron mode. The mass of each measured peptide fragment was assigned to the known mass of expected peptides deduced from the protein sequence.

Chemical modification of glutamic acid and aspartic acid residues. Modification of His6-Gir2$^{[1-265]}$ with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Novabiochem), in the presence of excess ethanolamine (Sigma), was a variation of previously described protocols [6–11]. To purified proteins or standard, at 1 mg/ml in 20 mM Tris–HCl, pH 8.0, the following was added: 500 mM ethanolamine (pH 6.0); 30 mM Mes buffer (pH 5.5); and 12 mM EDC. The reaction was allowed to proceed for 1–2 h at 23 °C and then stopped with Laemmli loading buffer. Samples of the reaction were subjected to SDS–PAGE (12%) and the molecular masses were determined by their relative mobilities (R_f).

Circular dichroism. His6-Gir2$^{[1-265]}$ at 0.6 mg/ml in 20 mM Tris–HCl, pH 8.0 (with 1.2% SDS or without SDS), was placed in a cuvette with 0.05 cm path length. Circular dichroism measurements were made on a JASCO-J810 spectropolarimeter equipped with a temperature control system in a continuous mode. Far-UV measurements (an average of five scans) were carried out over wavelengths 265–190 nm with 0.5 nm bandwidth, 1 s response time, and 20 nm/min scan speed at 23 °C unless otherwise specified. Spectra were corrected by subtraction of the buffer signal. Thermal behavior experiments were performed with a heating rate of 1 °C/min and a response time of 2 s. Molecular ellipticity, $[\theta]_{MRW}$, was calculated and the secondary structure was estimated from computer analysis using CONTIN/LL and SELCON3 softwares [13].

Results and discussion

Expression and purification of His-tagged Gir2

His-tagged proteins corresponding to the complete Gir2 sequence (amino acid residues 1–265) and a C-terminus truncated form (amino acid residues 1–166), which contains the RWD/GI domain and the acidic residues, were purified using nickel-chelating resin. SDS–PAGE analysis revealed that the apparent molecular mass was 63 kDa for His6-Gir2$^{[1-265]}$ instead of the expected 34.4 kDa, and 54 kDa for His6-Gir2 $^{[1-166]}$ instead of 23.9 kDa (Fig. 1). This result was reproduced using different denaturing gels (not shown). Cleavage of the proteins with thrombin to eliminate the His6-tag did not result in normal migration (Fig. 1). Considering the existence of three cysteine residues in the sequence of Gir2, we hypothesized that Gir2 could form a stable dimer in solution even after incubating in denaturing conditions of the Laemmli loading buffer. To test this, the purified proteins were incubated with 1–500 mM DTT, or with 7 M of 2-mercaptoethanol for 2 h prior to

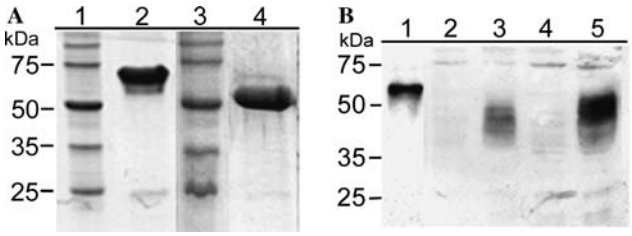

Fig. 1. Anomalous electrophoretic migration of Gir2. (A) *Escherichia coli* expressed recombinant His6-Gir2. 12% SDS–PAGE of His6-Gir2 purified on nickel-chelating resin. Lanes 1 and 3, molecular mass standards; lane 2, His6-Gir2$^{[1–265]}$; and lane 4, His6-Gir2$^{[1–166]}$. (B) Yeast expressed Gir2. Immunoblot using antiserum raised against Gir2. Lane 1, recombinant Gir2$^{[1–265]}$, cleaved with thrombin; lanes 2 and 4, yeast whole cell extracts (10 and 20 μg of total protein, respectively) from uninduced yeast cells; and lanes 3 and 5, whole cell extracts (10 and 20 μg of total protein, respectively) from cells grown with 10% galactose.

SDS–PAGE. The reduced proteins also migrated abnormally (data not shown). These results suggested the possibility that Gir2 could form a stable non-covalent dimer. Therefore, His6-Gir2 was incubated with high concentration of salts (2 M KCl or 3 M NaCl) or denaturing agents (8 M urea and 10% SDS) and then subjected to SDS–PAGE. The salt-treated or denatured protein migrated with the same abnormal Mr (data not shown).

In vivo expressed Gir2

In order to investigate whether the native Gir2 protein also behaves anomalously, we raised antibodies against the recombinant protein and used this serum in immunoblots of yeast whole cell extracts prepared from two isogenic strains, differing only in that one of them was deleted of the chromosomal GIR2 gene. The protein does not form a clearly defined band, migrating as a smear centered around 50 kDa and thus being difficult to identify in immunoblots (data not shown). To have a better resolution, we overexpressed Gir2 (residues 1–265), without the His-tag, in yeast cells from a galactose inducible promoter. As shown in Fig. 1, the protein migrates as a smear, at a position corresponding to 40–60 kDa. Thus, the behavior of the yeast expressed protein is analogous to the recombinant protein. Global analysis of protein expression in *S. cerevisiae*, in which the ORFs were tagged with a high affinity epitope (modified TAP) [14], showed that Gir2, expressed from its natural chromosomal location, displays a slow migration with a diffuse pattern in gradient gels [S. Ghaemmaghami, personal communication]. The lack of a clear definition in SDS–PAGE of Gir2 expressed in yeast may be related to the presence of a putative PEST sequence which may impart a high degradation rate to the protein in vivo.

Molecular mass determination and peptide mass fingerprinting by MALDI-TOF spectrometry

The molecular masses of purified His6-Gir2$^{[1–265]}$ and His6-Gir2$^{[1–166]}$ were determined using a MALDI-TOF/Pro mass spectrometer with a matrix of sinapinic acid. The mass assigned for the two recombinant His-tagged proteins corresponded exactly to those predicted from the primary sequence, as shown in Fig. 2 for His6-Gir2$^{[1–265]}$. The molecular masses were also determined by Q-TOF mass spectrometry and the results were the same as those obtained from MALDI-TOF (data not-shown).

Peptide mass fingerprinting analysis of His6-Gir2$^{[1–265]}$ was performed to ascertain the identity of the expressed protein. Twenty-one peptides were generated and eight of them were assigned to the known amino acid sequence of Gir2 with high probability (Fig. 2). Some of the other peptides could be due to contamination of the trypsin used. The results obtained confirmed that the recombinant protein with anomalous behavior in SDS–PAGE is in fact Gir2.

Electrophoretic mobility shift after chemical modification of acidic amino acids

The anomalous behavior of some proteins in SDS–PAGE has been shown to be corrected by the chemical modification of carboxylate groups of acidic amino acids in the presence of water-soluble carbodiimide and excess of an amine, which neutralizes the negative charges [6,7,11,15–17]. This technique is also used to access the functional relevance of acidic residues on a protein [8–10]. In order to determine if the high content of acidic residues of Gir2 (23.5% D + E, corresponding to a net charge of −26.1 at pH 7.0 for the His-tagged protein) was responsible for the anomalous migration, we converted the negatively charged amino acids of the purified protein into neutral ones, using water-soluble carbodiimide (EDC) in the presence of excess of ethanolamine. EDC-modified His6-Gir2$^{[1–265]}$ migrated with approximately the expected molecular weight, i.e., 34 kDa (Fig. 3). The same result was observed for His6-Gir2$^{[1–166]}$ (data not shown). Modification of His-tagged Gir2 resulted in an electrophoretic band which was much more diffuse than that of unmodified protein, possibly indicating different levels of esterification and/or some EDC-mediated intramolecular cross-linkage, which may diminish the frictional coefficient of the protein below the expected.

His-tagged Gir2 has a theoretical −26.1 net charge at pH 7.0 and we could infer that the protein would migrate faster than expected. However, the results shown here suggest that the high content of acidic amino acids might result in low binding of SDS producing insufficient denaturation and slow migration in the gel, as

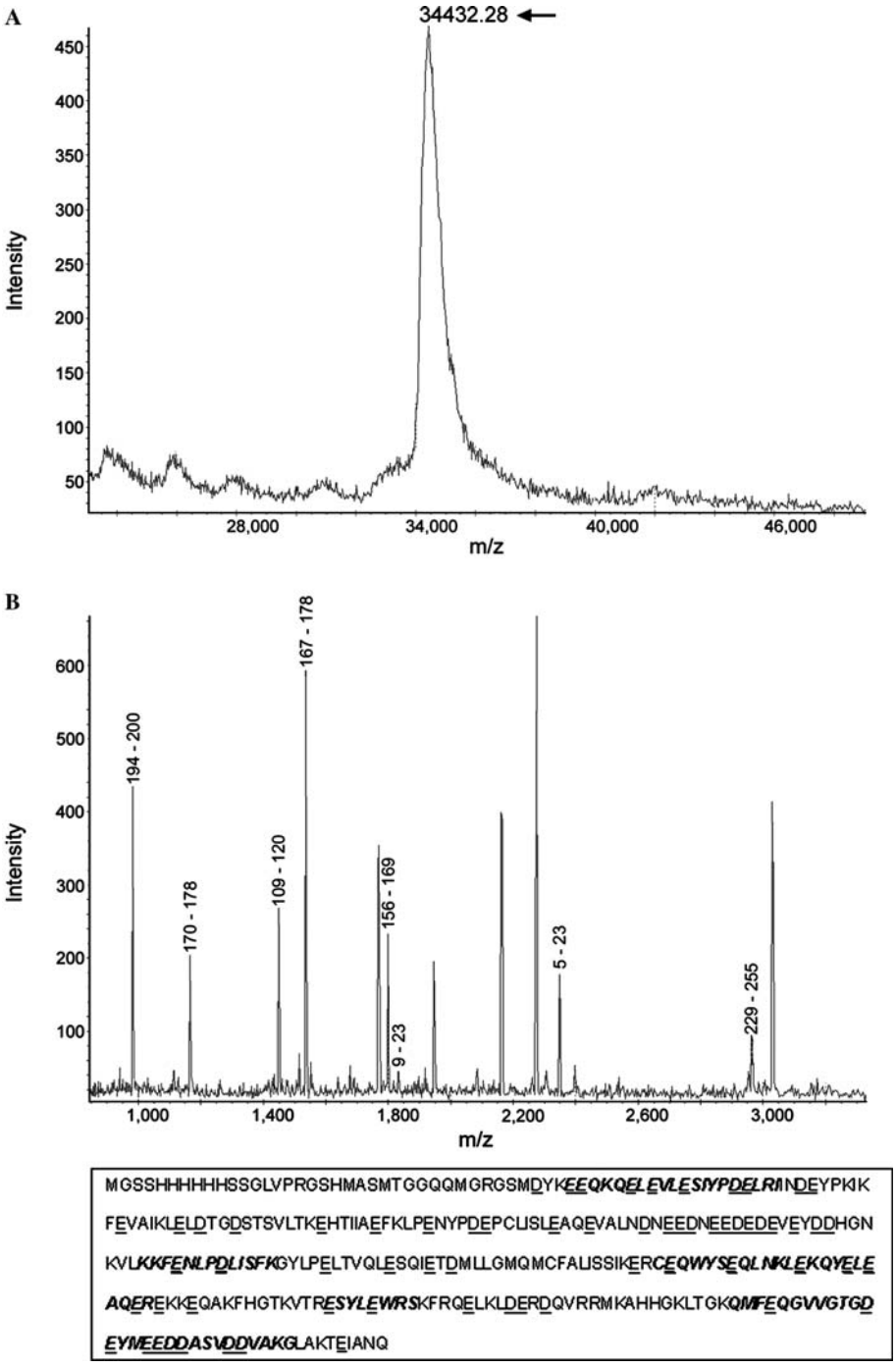

Fig. 2. Spectrometric analysis of His6-Gir2[1–265]. (A) Molecular mass derived from MALDI-TOF spectrometry. The arrow indicates the obtained mass. (B) Peptide mass fingerprinting (PMF) by MALDI-TOF spectrometry. Upper panel, mass determination of peptides from trypsin digestion of His6-Gir2 with the numbers above the peaks indicating the amino acid residues of the native protein; lower panel, amino acid sequence of His6-Gir2 showing the assigned peptides in PMF (bold) and acidic amino acids (underlined).

previously found for several prokaryotic and eukaryotic proteins. In addition, the protein slow migration could be due to a large asymmetry producing an increased frictional resistance in polyacrylamide gel electrophoresis. The exact explanation for the influence of negative charged residues in reducing the electrophoretic mobility of proteins remains unclear.

Molecular weight estimation by size exclusion chromatography

In order to further investigate the basis of the behavior of this protein, we performed gel filtration analysis using a Superdex 200 HR column, and bovine serum albumin and lysozyme as standards. Gir2 eluted

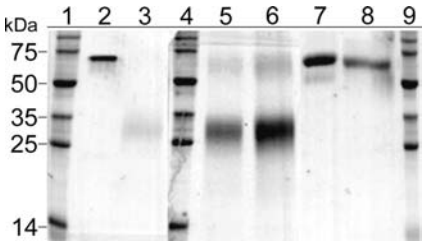

Fig. 3. Chemical modification of His6-Gir2[1–265]. 12% SDS–PAGE. Lanes 1, 4, and 9, molecular mass standards; lane 2, unmodified Gir2 (2 μg); lane 3, EDC-modified Gir2 (2 μg); lanes 5 and 6, EDC-modified Gir2 (6 and 12 μg, respectively) in excess of ethanolamine; lane 7, unmodified BSA (4 μg) (apparent MW 63 kDa); and lane 8, EDC-modified BSA (4 μg) in excess ethanolamine.

with an increased size, 62 kDa for the full length protein (amino acids 1–265) and 60 kDa for the truncated protein (amino acids 1–166) (Table 1). We performed the same experiment in the presence of 2 M KCl for disruption of possible non-covalent dimers, and still the protein eluted with the same M_r (data not shown). These results discarded the possibility that the high retention rate of Gir2 might be due to the formation of stable dimers. Indeed, DLS (Dynamic Light Scattering) in non-denaturing conditions suggested that this protein was present as a monomer in solution (data not shown). The inferred Stokes radius of His6-Gir2[1–265] was calculated to be 3.23 nm, which deviates 23% from that predicted from the sequence. This determination was made using an empirical equation which considers the relative molecular weight obtained in gel filtration experiments (Table 1) [5]. Considering these results, we suggest that Gir2 is a non-globular protein and probably has an extended structure.

Recent analysis established a clear relationship between molecular weight of proteins from different conformational class and their Stokes radii [18]. For comparison, we calculated the predicted Stokes radius (R_s) for monomeric His-tagged Gir2 (34 kDa) in the five known protein conformations: folded, molten-globule, pre-molten globule, natively unfolded "pre-molten globule-like," and natively unfolded "coil-like," as described in [18] (Table 2). The similarity between the predicted R_s by gel filtration experiments (3.23 nm for His6-Gir2[1–265] and 3.18 nm for His6-Gir2[1–166]) and that predicted from conformational class (3.67 nm for His6-Gir2[1–265] and 3.21 nm for His6-Gir2[1–166]) suggests that this protein is a pre-molten globule (PMG).

Table 2
Theoretical R_s for His-tagged Gir2 in different conformation states

Conformational state	R_s (nm)	
	His6-Gir2[1–265]	His6-Gir2[1–166]
Natively folded	2.62	2.32
Molten globule	2.88	2.56
Pre-molten globule (PMG)	3.67	3.21
Natively unfolded (coil-like)	4.81	4.04
Natively unfolded (PMG-like)	3.86	3.35

R_s, Stokes radii.

Estimation of the secondary structure by circular dichroism

Circular dichroism spectrum (scanned from 265 to 190 nm) was used to estimate the secondary structure present in His6-Gir2[1–265] using CONTIN/LL and SELCON3 programs. By this approach, this protein has a content of 16.4% α-helix and 30.5% random coils, in non-denaturing conditions. Far-UV measurements in the presence of SDS and temperature variations monitored at 222 nm showed that the α-helix content in His6-Gir2 decreased only slightly (~2.8%) at 70 °C and the secondary structure predictions showed that the content of random coils was maintained during thermal increases (Fig. 4). Thus, His-tagged Gir2 behaves as a thermostable protein, at least up to 70 °C. These data suggest that the anomalous electrophoretic behavior could be due to persistent structure in conjunction with a high content of random coils and acidic amino acids. This possibility would cause the protein to behave as an extended molecule in which the conformation is stabilized by the repulsion between a large number of negatively charged residues. Then, the anomalous electrophoretic behavior is not merely a charge effect. A similar mechanism to explain this behavior has been previously demonstrated for another protein [19].

It is now recognized that many protein domains, or even full-length proteins, are intrinsically unstructured. Local or global disorders in proteins appear to be quite

Table 1
Molecular determinations for Gir2

	Molecular mass (kDa)				R_s	
	Predicted	Observed			Predicted	Observed
		SDS–PAGE	GF	MS		
Gir2 (native)	30.9	48*	—	—	2.53	—
His6-Gir2[1–265]	34.4	63	62	34.4	2.62	3.23
His6-Gir2[1–166]	23.9	54	60	23.9	—	3.18
His6-Gir2[1–265] EDC-modified	34.4	30*	—	—	—	—

R_s, Stokes radius (nm); GF, Gel filtration; MS, Mass spectrometry; and * mean molecular mass.

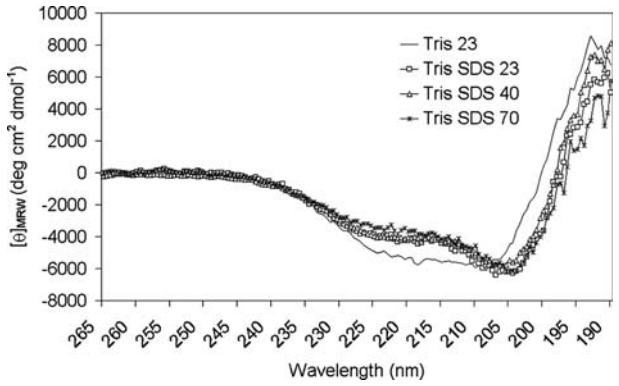

Fig. 4. CD spectra of His6-Gir2[1–265]. Far-UV CD spectra monitored between 265 and 190 nm in 20 mM Tris–HCl, pH 8.0, in the absence or presence of 1.2% SDS, at 23, 40, and 70 °C as indicated in the legend.

prevalent in the various genomes. Spectroscopic methods have shown that numerous proteins appear to be unfolded or only partially folded under normal conditions [12].

We propose that Gir2 is a "natively unfolded" protein, probably belonging to the group of intrinsic premolten globule, based on the following diagnostic properties: (1) stability to heat denaturation; (2) a highly charged (often acidic) amino acid sequence; (3) "random coil" structure; (4) an abnormally high Stokes radius; and (5) abnormal SDS binding leading to an unusual mobility in SDS–PAGE [18,20].

It has been recently proposed that unfolded proteins are subjected to a rapid turnover in the cell mediated by a PEST sequence motif, which is very often present in unstructured regions of proteins targeted for rapid degradation [12]. Interestingly, Gir2 also has a PEST sequence (amino acids 62–102) with a very high score of +12,3, as predicted by the PESTfind algorithm [2]. The behavior of Gir2 in vivo might be related to this sequence.

This is the first report on the highly anomalous behavior of an acidic protein of the yeast *S. cerevisiae*. Probably more proteins with these characteristics exist in yeast but are still uncharacterized.

Acknowledgments

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