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The Expanding Landscape of Moonlighting Proteins in Yeasts

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SUMMARY

Moonlighting proteins are multifunctional proteins that participate in unrelated biological processes and that are not the result of gene fusion. A certain number of these proteins have been characterized in yeasts, and the easy genetic manipulation of these microorganisms has been useful for a thorough analysis of some cases of moonlighting. As the awareness of the moonlighting phenomenon has increased, a growing number of these proteins are being uncovered. In this review, we present a crop of newly identified moonlighting proteins from yeasts and discuss the experimental evidence that qualifies them to be classified as such. The variety of moonlighting functions encompassed by the proteins considered extends from control of transcription to DNA repair or binding to plasminogen. We also discuss several questions pertaining to the moonlighting condition in general. The cases presented show that yeasts are important organisms to be used as tools to understand different aspects of moonlighting proteins.

INTRODUCTION

Proteins have been described as "the most versatile class of biomolecules" (1). An unexpected facet of this versatility emerged when it was shown that one protein could be used by cells to perform completely different, unrelated, functions. Piatigorsky et al. (2) demonstrated that duck δ-crystallin and argininosuccinate lyase were encoded by the same gene and termed this phenomenon gene sharing (3). Further extensive studies with crystallins from different species revealed that many of them were identical to proteins that had been identified as having a different metabolic function (4). Other cases of proteins playing dual roles continued to appear in the literature, and their number has soared in recent years (5). The term gene sharing, first proposed for these proteins (3), may present some ambiguities, as there are cases in which a gene may encode different protein forms. In yeasts, this can be due to different sites for initiation of translation (6, 7), termination (8), or splicing (9). In these cases, the corresponding protein forms also share a gene but this gene sharing is not the same as that of a single protein playing two different roles. The alternative designation "moonlighting proteins" was proposed to name those proteins, and a clear-cut definition to delimit which proteins may be considered as such was also provided (10). The limits imposed by the definition were that the diverse functions of moonlighting proteins shall not be the result of a gene fusion event, or of differential gene splicing; proteins with a single function in different subcellular localizations were excluded from the

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TABLE 1 Some moonlighting proteins in yeasts

Protein	Canonical function	Moonlighting activity	Yeast species	Reference(s)
Homocitrate synthase	Acyl transferase (lysine biosynthesis)	Repair of damaged DNA	*S. cerevisiae*	29, 32
Superoxide dismutase	Conversion of superoxide radical into O_2 and H_2O_2	Regulation of genes related to oxidative stress	*S. cerevisiae*	42
Pyruvate decarboxylase	Pyruvate decarboxylation (glycolysis)	Regulation of *PDC1*/*PDC5* transcription	*S. cerevisiae*, *K. lactis*	47, 49, 50, 52
Zuotin	Molecular chaperone	Activation of pleiotropic drug resistance	*S. cerevisiae*	58, 60, 64
Subunit Sdh3 of succinate dehydrogenase	Oxidation of succinate (citric acid cycle), electron transport	Assembly of mitochondrial translocase	*S. cerevisiae*	70
Enolase	Dehydration of 2-P-glycerate (glycolysis)	Binding to plasminogen	*C. albicans*	79
Phosphate-glycerate mutase	Isomerization of 3-P-glycerate (glycolysis)	Binding to plasminogen and to complement regulators	*C. albicans*	78, 81
Glycerol-3-phosphate dehydrogenase	Reduction of dihydroxyacetone phosphate to glycerol-3-phosphate (ancillary to glycolysis)	Binding to plasminogen and to complement regulators	*C. albicans*	84
Hgt1	Glucose transporter (glycolysis)	Binding to complement regulators	*C. albicans*	90
Hal3	Decarboxylation of phosphate-pantothenoyl–L-cysteine (CoA biosynthesis)	Inhibitor of phosphatases Ppz1 and Ppz2	*S. cerevisiae*, *S. pombe*	95, 99

[a] Shown are the moonlighting proteins considered in the text. A table with other moonlighting proteins from yeasts is presented in reference 22.

group (10). Chapple and Brun (11) have proposed to expand this definition to cover all proteins that perform dissimilar functions, therefore including proteins where separate domains are responsible for their multifunctionality. Structural proteins that serve as scaffolds and interact with different proteins are generally not considered moonlighting proteins (12), and this criterion is followed in this article. Secreted proteins are accepted in this category, as the evidence of their numerous roles in different organisms is well documented (12–15). It may be noted that the word "moonlighting" had been used already in 1978 by Freedman to refer to multifunctional proteins in an article entitled "Moonlighting Molecules" (16). Moonlighting proteins are found in diverse organisms, and current dedicated databases list several hundred of them, distributed among the different biological kingdoms (5, 17). Their noncanonical roles cover a bewildering array of functions such as control of transcription, protection of DNA, assembly of organelles, chaperoning, splicing of introns, or binding to plasminogen (12, 18–20). Such alternative functions often cause unexpected phenotypes in monogenic mutants, and it has been proposed that the complex phenotypes of some single-gene disorders could be due to unknown moonlighting functions of the main protein concerned (21).

Many moonlighting proteins are present in different yeast species, and their roles are very diverse; for example, hexokinase 2 and galactokinase act as transcriptional regulators, aconitase functions in mitochondrial DNA maintenance, and aldolase and pyruvate carboxylase participate in the assembly of some vacuolar or peroxisomal enzymes (18, 22). While most of these proteins have been described in *Saccharomyces cerevisiae*, this probably reflects the starring role of this yeast in research and not some basic difference between species. As awareness of the existence of moonlighting proteins has spread, more cases are being described. Since the publication of the last comprehensive review devoted to moonlighting proteins in yeasts (22), new, well-documented cases have appeared that increase the number of yeast proteins belonging to this group (Table 1).

In this article, we present several newcomers to the class of yeast moonlighting proteins, examining the experimental support to qualify them as such. The wide importance of the moonlighting phenomenon for yeast research, both basic and applied, is also discussed.

HOMOCITRATE SYNTHASE PARTICIPATES IN DNA REPAIR

The amino acid lysine plays a particular role in proteins, as its ε-amino group can be modified by different substituents, thereby altering the activity of the corresponding protein. Yeasts and other fungi synthesize lysine by the so-called α-aminoadipate pathway that starts with a condensation of α-oxoglutarate, an intermediate of the tricarboxylic acid cycle, and acetyl coenzyme A (acetyl-CoA) to produce homocitrate. This reaction is catalyzed by the enzyme homocitrate synthase. Tucci and Ceci (23) undertook the purification of the corresponding protein from *S. cerevisiae* and showed the existence of two enzymes by isoelectric focusing; both of them were inhibited by lysine, although not to the same extent. Ramos et al. (24) isolated a fragment of yeast DNA whose disruption greatly reduced homocitrate synthase activity; they called the corresponding gene *LYS20* and demonstrated that it encoded the isoenzyme responsible for about 70% of lysine production, thus confirming the existence of another protein with homocitrate synthase activity. The corresponding gene, *LYS21*, was later identified by Feller et al. (25), and the protein was shown to be the main homocitrate synthase activity during growth in ethanol (26); it was also shown that Lys20 and Lys21 present different kinetic and regulatory properties (26).

Since it had been reported that not all of the steps in the biosynthesis of lysine took place in the same subcellular compartment (27), Chen et al. (28) tried to determine the localization of homocitrate synthase. Using monoclonal antibodies against nuclear proteins from *S. cerevisiae*, they showed that the majority of homocitrate synthase was present in the nucleus. Later on, this result was confirmed by using green fluorescent protein (GFP) fusions to Lys20 and Lys21 (25). The unexpected localization led Chen et al. (28) to raise provocative questions about the significance of the nuclear localization of the enzyme and its possible enzymatic activity in the nucleus. They speculated that in the nucleus, homocitrate synthase could have a function not directly related to lysine biogenesis, playing a role in nuclear structure and/or function. The work of Scott and Pillus (29) has shown that these questions were pertinent. In fact, Lys20 performs a function in the nucleus that is not related to lysine synthesis; it participates in the damaged DNA repair process. This was found during studies of an *esa1-414* mutant affected in the activity of Esa1, an essential histone acetyltransferase in yeast (30). This mutant is impaired in the repair of DNA double-strand breaks and therefore does not grow in the presence of camptothecin, a drug that interferes with toposisomerase I (31). Looking for suppressor genes that restored growth to *esa1-414* mutants on camptothecin, Scott and Pillus (29) identified *LYS20*; *LYS21* showed a much weaker suppressor effect. The authors also showed that some *lys20* mutations that abolished homocitrate synthase activity were still able to accomplish their function in DNA repair. Using homocitrate synthase versions that conserved catalytic activity but were unable either to enter the nucleus or to remain in it, the same authors (29) found that they suppressed camptothecin toxicity toward *esa1-414* mutant cells less efficiently than the wild-type enzyme, thus showing that the nuclear localization of homocitrate synthase was critical for its nonmetabolic role.

Following this work, Torres-Machorro et al. (32) undertook the characterization of the mechanism of suppression of *esa1* mutations by overexpression of *LYS20*. Testing versions of Lys20 with mutations in different amino acids and domains, they determined that the domain of Lys20 implicated in the moonlighting DNA repair function was located in the C-terminal portion of the protein, in front of a nuclear localization signal previously identified by Scott and Pillus (29); such a domain was not found in Lys21. By using chromatin immunoprecipitation preceded by a cross-linking step, it was found that Lys20 was enriched in the chromatin fraction (32). This association, however, was not detected if cross-linking was omitted, suggesting that the interaction of Lys20 with chromatin was either transient or unstable. It was also observed that Lys20 was recruited to DNA double-strand breaks and that overexpression of *LYS20* caused larger amounts of Lys20 to appear at the breaks. A mutated form of Lys20 affected in the moonlighting domain was recruited at the breaks as well as a wild-type version of Lys20, but it failed to correct the defects in DNA repair that occur in *esa1* mutants.

Repair of DNA double-stranded breaks is dependent on histone eviction at the damage sites, a process required for further reactions in the DNA repair pathway (33). Histone eviction, in turn, is facilitated by Esa1, which transiently acetylates histone H4 at the DNA breaks (34), and by the chromatin remodeling complex INO80 (35). Lys20 could therefore promote the recruitment at the breaks of either Esa1-414 or the INO80 complex. It has been found that Lys20 overexpression does not facilitate the binding of Esa1-414 at the damage sites but suppresses *esa1* mutations by increasing the amount of the INO80 complex that accumulates at the DNA breaks (32).

SUPEROXIDE DISMUTASE Sod1 CONTROLS TRANSCRIPTION OF GENES ENCODING PROTEINS IMPLICATED IN OXIDATIVE RESPONSE AND DNA REPAIR

Organisms are exposed to injuries caused by reactive oxygen species (ROS) originated both intra- and extracellularly. ROS are potent oxidants that can damage all types of cellular components; to minimize the damage, several defense mechanisms have been selected in the course of evolution. Among the agents involved in those mechanisms are the superoxide dismutases, which catalyze the conversion of the superoxide radical O_2^- into O_2 and H_2O_2. The latter molecule can, in turn, be converted into H_2O and O_2 by catalases. *S. cerevisiae* possesses two forms of superoxide dismutase, Sod1, which is cytoplasmic, and Sod2, which is mitochondrial. Sod1 is a Cu-Zn-containing enzyme, while Sod2 is a Mn-containing form. Yeast mutants affected in Sod1 present multiple phenotypes such as methionine auxotrophy, slow growth, loss of viability in stationary phase, or a decreased replicative life span (36–40). Mutants lacking Sod2 also show different phenotypes, among them decreased survival in the stationary phase (37).

While studying the regulation of yeast Sod1 under oxidative stress produced by 4-nitroquinoline-*N*-oxide, an oxygen radical-producing compound (41), Tsang et al. (42) did not observe changes in Sod1 activity; however, by using fluorescence microscopy, they noticed a change in the subcellular localization of Sod1. In untreated cells, Sod1 is basically cytoplasmic, while treatment with 4-nitroquinoline-*N*-oxide made this protein mainly nuclear. This change was also observed in response to another ROS-generating compound, H_2O_2, and was confirmed by subcellular fractionation of the treated cells. Experiments using different yeast mutants that spontaneously produce a high level of ROS showed that the change in localization could also take place in the absence of an external treatment, demonstrating that it also occurred when ROS were generated intracellularly.

An important consequence of oxidative stress is DNA damage that may be corrected by the DNA damage checkpoint pathway. The transmission of the DNA damage signal through a cascade of protein kinases, including Mec1 and Dun1, is affected in *sod1* mutants, and this results in problems in coping with oxidative stress (36). Interestingly, the nuclear localization of Sod1 in response to ROS is blocked in the absence of Mec1 or Dun1 (42). While previous studies had shown that Sod1 and Dun1 could form a complex (43), Tsang et al. (42) found that oxidative conditions enhanced this interaction and that deletion of Dun1 blocked Sod1 in the cytoplasm. They also found that Dun1 phosphorylates Sod1 at serine residues S60 and S99 and that this phosphorylation contributed to its nuclear localization. Suspecting a regulatory role for nuclear Sod1, Tsang et al. (42) studied the effects of treatment with H_2O_2, the product of Sod1, in a wild-type strain and in a *sod1* mutant by DNA microarray analysis. They found that induction by H_2O_2 of >100 genes related to different responses to oxidative stress was significantly attenuated in the *sod1* mutant. Chromatin immunoprecipitation experiments showed binding of Sod1 to the promoters of two stress genes that require Sod1 for their activation but not to the promoter of *ACT1*, a control gene independent of Sod1. These results suggest an important role for Sod1 as a direct regulator of the transcription of

genes related to oxidative stress, and it appears likely that Sod1 acts as a moonlighting protein. It should be noted, however, that it has not yet been established whether the catalytic activity of Sod1 is required for its regulatory function.

A role for Sod1 in the process of glucose repression in *S. cerevisiae* has also been described by Reddi and Culotta (44), who found that in *sod1* mutants, glucose did not repress respiration. Sod1 acts by binding the protein kinases Yck1 and Yck2 and stabilizing them, thus allowing the repression of respiration by glucose. However, their stabilization requires the catalytic activity of Sod1, as it depends on the formation of H_2O_2; therefore, it is not considered a moonlighting function here (44).

PYRUVATE DECARBOXYLASE REGULATES THE TRANSCRIPTION OF ITS ENCODING GENE

In yeasts able to ferment sugars, pyruvate produced in glycolysis is located at a branching point between fermentative and oxidative metabolism. The enzymes pyruvate decarboxylase (Pdc) and pyruvate dehydrogenase, which initiate the respective metabolic branches, are finely regulated (45). Pdc, in the first step of the fermentative branch, catalyzes de decarboxylation of pyruvate to acetaldehyde. In *S. cerevisiae*, there are two genes, *PDC1* and *PDC5*, that encode the bulk of the Pdc activity, with *PDC1* accounting for about 90% of the protein and *PDC5* accounting for about 10% (46). The expression of these genes is controlled by the carbon source available; during growth on glucose, there are high levels of Pdc1, while Pdc5 is almost undetectable (47). Although Schmitt and Zimmermann (48) isolated mutants devoid of pyruvate carboxylase activity that did not grow in glucose, Hohmann and Cederberg (47) found that a *pdc1*Δ mutant grew in glucose and exhibited elevated levels of Pdc. They showed that this was due to the increased expression of *PDC5*, which is scarcely expressed in wild-type strains, and called this type of regulation autoregulation. Eberhardt et al. (49) found that the initial mutants isolated by Schmitt and Zimmermann (48) abolished growth in glucose because they expressed a form of Pdc1 that lacked catalytic activity but was able to reduce *PDC5* expression. Pdc1 also affected its own transcription, as shown by Liesen et al. (50), who observed that in a strain carrying a *PDC1* deletion, the expression of the *Escherichia coli lacZ* gene under the control of the *S. cerevisiae PDC1* promoter was increased 6-fold. They also found that in a mutant with the *PDC1* gene deleted, expression of *PDC1* under the control of the *TPI* promoter repressed the expression of *PDC5*. The results available indicate that autoregulation is due to an action of the Pdc1 protein not related to its catalytic activity. Deletion of *PDC5* did not produce similar effects, indicating a specific role for Pdc1.

At least two possibilities may be contemplated regarding the mechanism underlying the regulatory role of Pdc1; Pdc1 could act directly as a repressor on the promoters of *PDC1* and *PDC5*, or it may interfere with an activator of the transcription of these genes. Although Pdc1 has been found both in the cytoplasm and in the nuclei of yeast cells (51), there is as yet no evidence of its binding to DNA. Liesen et al. (50) presented a model in which a protein (Era) would interact with the *ERA* sites of the promoter of *PDC1* and with a hypothetical activating protein whose activation capacity would depend on the concentration of Pdc1 (Fig. 1A). The activating protein could be Pdc2, a regulatory protein needed for high *PDC1* expression (51). This model has received indirect support from results obtained with the respirofermentative yeast *Kluyveromyces lactis* (52). *K. lactis* has a single gene encoding Pdc,

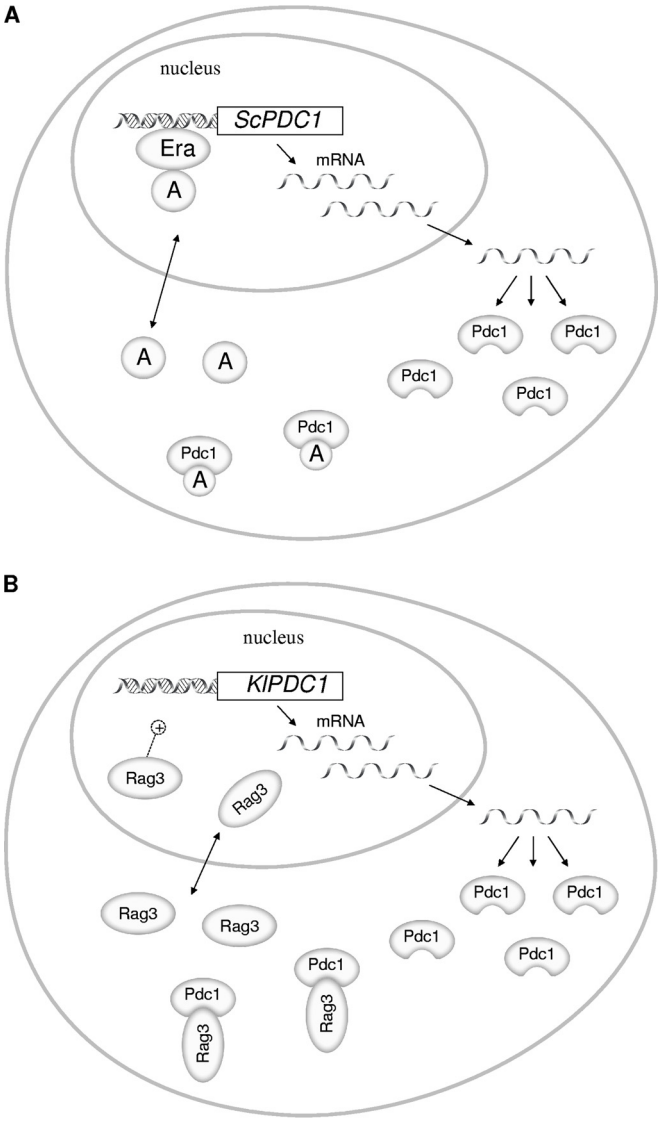

FIG 1 Scheme for autoregulation of Pdc1 synthesis. (A) In *S. cerevisiae*, a hypothetical protein, Era, together with an activating protein A (Pdc2?), activates the transcription of *PDC1*. The Pdc1 protein in the cytosol would sequester the activating protein and decrease *PDC1* transcription (50). (B) In *K. lactis*, transcription of Kl*PDC1* is activated by the regulatory protein Rag3, an ortholog of Pdc2. When the concentration of Pdc1 is high, most of the Rag3 protein is bound to it and trapped in the cytosol; consequently, the rate of Kl*PDC1* transcription is low. When Pdc1 levels decrease, Rag3 is liberated and can enter the nucleus, activating Kl*PDC1* transcription (52).

Kl*PDC1*, that appears to be also autoregulated since a Kl*PDC1-lacZ* fusion gene is strongly derepressed in a *K. lactis* mutant devoid of Pdc1 (53). ScPdc1 may replace KlPdc1, and expression of an *S. cerevisiae PDC1* gene bearing a point mutation that produces an inactive Pdc1 (54) caused a decrease in KlPdc1 transcription (53), suggesting a mechanism for autoregulation in *K. lactis* similar to that found in *S. cerevisiae*. Ottaviano et al. (52), using a *K. lactis* strain with Kl*PDC1* deleted and harboring a Kl*PDC1-lacZ* fusion gene, searched for mutants that abolished β-galactosidase production. One such mutant turned out to harbor a mutation in *RAG3*, a gene encoding a protein with important sequence similarity to the Pdc2 protein of *S. cerevisiae* (55). A two-hybrid assay

showed interaction between Rag3 and KlPdc1, an interaction confirmed by coimmunoprecipitation experiments. Subcellular localization experiments showed that in glucose-grown cultures, KlPdc1 had a cytoplasmic localization and was not detected in the nucleus, while Rag3 showed a uniform distribution in the cell; in the absence of KlPdc1, Rag3 became localized predominantly in the nucleus. Pdc1 itself was not detected in the nucleus. The authors proposed a model (Fig. 1B) in which autoregulation is effected by an interaction between KlPdc1 and Rag3 that would sequester Rag3 in the cytoplasm. Abolition of this interaction will direct Rag3 to the nucleus, allowing increased transcription of KlPdc1 through direct or indirect action on the Kl*PDC1* promoter (52).

ZUOTIN, A COCHAPERONE THAT ACTIVATES A TRANSCRIPTION FACTOR

Zuotin was identified in nuclear extracts from *S. cerevisiae* by Zhang et al. (56) in a search for proteins binding to Z-DNA, the left-handed helix form of DNA. Its name is derived from the word *zuo*, left in Chinese (56). Shortly thereafter, while looking for tRNA-binding proteins in the nucleus of *S. cerevisiae*, Wilhelm et al. (57) isolated a protein that turned out to be zuotin and suggested a possible role for it in the processing or transport of tRNA to the cytoplasm. Zuotin has also been found to be associated with ribosomes by centrifugation through sucrose density gradients (58) and to coimmunoprecipitate with Pdr13 (59). Pdr13, also called Ssz1, is a chaperone that enhances the activity of the transcription factor Pdr1, which is involved in pleiotropic drug resistance (PDR) (60, 61). These observations suggest that Zuo1 may play different functional roles, and this is supported by the fact that the protein is found in different cellular compartments and that diverse *zuo1* mutants do not present the same phenotype, as described below.

While Zuo1 was first identified in yeast nuclear extracts (56, 57), Yan et al. (58), using a functional Zuo1-GFP fusion, found that Zuo1 had a predominantly cytosolic localization. Later experiments carried out during a study of ribosome biogenesis demonstrated that Zuo1 had both a nuclear and a cytoplasmic localization (62). Deletion of *ZUO1* produced a phenotype of slow growth at low temperature and sensitivity to salt, similar to that produced by double deletion of the chaperones Ssb1 and Ssb2. A *zuo1 ssb1 ssb2* triple mutant did not present a stronger phenotype, showing that the mutations had no additive effects (58). Ssb proteins are molecular chaperones of the Hsp70 class that act in the early folding steps during protein biogenesis (63). Since the folding process is facilitated by the interaction of chaperones with DnaJ proteins and Zuo1 has the hexapeptide motif KYHPDK, which is also present in the DnaJ protein of *E. coli*, Yan et al. (58) proposed that Zuo1 could function together with Ssb proteins in the ribosome. They constructed a series of gene truncations and deduced that the charged region of the protein between amino acids 285 and 364 was critical for the interaction with the ribosome and that the C-terminal sequence was not necessary in the assays used.

In relation to another possible function of zuotin, the results of Eisenman and Craig (64) were important. While investigating mechanisms enhancing PDR, they found that, to activate PDR, Ssz1 needed to dissociate from the ribosomes. Following this result and considering that Ssz1 and Zuo1 form a 1:1 complex (65), the authors studied the effect of disruption of the association of Zuo1 with ribosomes on PDR. This was done with a mutant form of Zuo1 with an internal deletion of amino acids 285 to 364 that abolishes this interaction, and it was observed that resistance to antibiotics increased in this mutant. By constructing different partial deletions of *ZUO1*, it was established that the C-terminal domain of Zuo1 was necessary and sufficient for PDR activation. Using combinations of different mutations in *SSZ1* and *ZUO1*, Eiseman and Craig (64) showed that PDR activation can be produced by Ssz1 or Zuo1 independently and that this activation does not require their chaperone activity.

Molecular details of the activation of Pdr1 by Zuo1 have been worked out by Ducett et al. (60), who showed that simple dissociation of Zuo1 from the ribosome was not enough to produce activation of Pdr1. They found that expression of the C-terminal fragment containing amino acids 365 to 443 allowed yeast cells to resist cycloheximide, a compound extruded from the cell by a protein whose expression is upregulated by Zuo1-activated Pdr1. Since expression of the C-terminal fragment including amino acids 348 to 443 did not allow growth in the presence of cycloheximide, an inhibitory role for a region including amino acids upstream of position 365 was suggested.

Further experiments demonstrated that the fragment including amino acids 348 to 443 formed a four-helix bundle with residues 348 to 364 located in helix 1. *In vivo* assays showed that resistance to cycloheximide was observed only when constructions bearing mutations that destabilize helix 1 were expressed. It appears, therefore, that Zuo1 in the correctly folded conformation associates with the ribosome and that unfolding produces release from it and the ability to activate Pdr1.

The different results show that zuotin acts as a moonlighting protein; it facilitates protein folding through its interaction with Ssb proteins on the ribosomes and participates in PDR by activating the transcription factor Pdr1. Although it has been suggested that zuotin may process tRNA or transport it to the cytoplasm (57), this idea has not been explored further.

Sdh3 PARTICIPATES IN ELECTRON TRANSFER AND IN MITOCHONDRIAL TRANSLOCASE ASSEMBLY

Succinate dehydrogenase (SDH) participates in the tricarboxylic acid cycle in the oxidation of succinate to fumarate coupled with the transfer of electrons to ubiquinone. SDH is an iron-sulfur flavoprotein located in the mitochondrial inner membrane and is composed of four subunits, Sdh1 to Sdh4. Sdh1 and Sdh2 form the active site and carry the flavin adenine dinucleotide and iron-sulfur clusters, while Sdh3 and Sdh4 are needed for ubiquinone reduction (for a review, see reference 66). Like most mitochondrial proteins, the Sdh subunits are encoded by nuclear genes and synthesized as precursors in the cytosol. Import of cytosolic protein precursors into the corresponding mitochondrial space involves different translocases; specifically, proteins directed to the mitochondrial inner membrane that do not have a cleavable signal peptide are translocated by the TIM22 complex formed by various proteins, among them Tim18 (67). The amino acid sequence of Tim18 shows 39% identity and 58% similarity to that of Sdh4 (66, 68, 69); however, Sdh4 could not substitute for Tim18 (68). Although Sdh3 has no sequence homology with Tim18, during a genetic study of the elements of the TIM22 complex, Gebert et al. (70) found that overexpression of Sdh3 could complement the defect for growth on glycerol of a temperature-sensitive TIM22 mutant at the restrictive temperature. They also showed that in an *sdh3* mutant, the levels of different mitochondrial proteins were

greatly reduced. In addition, by blue native electrophoresis (71), it was observed that in the mitochondria of *sdh3* mutants, the TIM 22 complex was less abundant than in the wild type and showed a lower molecular mass of 250 kDa instead of the 300 kDa of the wild type (70). None of these alterations were observed in an *sdh4* mutant.

Import of the Tim18 precursor into mitochondria isolated from an *sdh3* mutant was not severely affected, but it could not be assembled into the TIM22 complex. This assembly was possible if the Sdh3 precursor was present in the incubation mixture, thus showing that Sdh3 is required for assembly of Tim18 into the TIM22 complex. Besides, an antibody shift analysis showed Sdh3 bound to Tim18 as an intermediate during the assembly of the TIM22 complex. Assembly of other proteins in the complex was also affected in *sdh3* mutants.

Purification of the TIM22 complex by using a tag linked to Tim18 and analysis of the recovered proteins showed the presence of Sdh3 but not that of other SDH subunits (70). Purification of the SDH complex using tagged Sdh4 yielded the components of the SDH complex, with Sdh3 among them. These results indicate that Sdh3 is present in both the SDH and TIM22 complexes, and they were confirmed by further experiments. When mitochondrial proteins separated by blue native electrophoresis were exposed to antibodies to Sdh3, two complexes were identified with sizes of 200 and 300 kDa, corresponding to those of SDH and TIM22, respectively. The functional presence of Sdh3 in two complexes with very different roles, electron transfer and protein assembly, qualifies Sdh3 as a *bone fide* moonlighting protein.

ENZYMES RELATED TO GLYCOLYSIS THAT BIND PLASMINOGEN OR COMPLEMENT REGULATORS

During the study of different bacterial infections, several proteins well characterized biochemically as cytosolic enzymes were found extracellularly or at the bacterial surface. Although these findings were initially considered artifactual, later research has established that these proteins are part of a network of virulence factors (13, 72). As the study of infections caused by yeasts has progressed, a number of glycolytic enzymes have been found that could have important functions as virulence factors. The extracellular glycolytic enzymes described as moonlighters in yeasts usually act through their binding to plasminogen or to complement regulators.

Enzymes Binding Plasminogen

Plasminogen is a mammalian protein that may be converted to plasmin, a serine protease that participates in different events such as fibrinolysis or degradation of extracellular matrix components (73). Plasminogen is recruited by different pathogens to facilitate host invasion (74), and this appears also to be the case for the opportunistic pathogenic yeast *Candida albicans*. *C. albicans* secretes a considerable number of proteins, several of which are involved in the invasion process (14, 75). Many of those proteins are secreted not by the standard endoplasmic reticulum-Golgi pathway but by other nonconventional mechanisms (76, 77). In the cell wall of *C. albicans*, Crowe et al. (78) identified eight proteins that bound plasminogen with high affinity; among them were five glycolytic enzymes: fructose bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, and alcohol dehydrogenase. Other studies have found that plasminogen can also bind enolase (79) and glycerol-3-phosphate dehydrogenase from *C. albicans*. The interaction of some of these proteins with plasminogen has been studied in some detail, as described in the following paragraphs.

Enolase. Enolase catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate in the glycolytic pathway. Jong et al. (79) found that *C. albicans* cells were able to bind plasminogen and plasmin and that purified enolase bound to a column or fixed to microtiter plates also interacted with those proteins. Lysine was a competitive inhibitor of this interaction, indicating that it depended on the lysine-binding domains of plasminogen (80). Besides, binding of plasminogen to enolase increased its affinity for its activator streptokinase, as occurs with its binding to other receptors. In an *in vitro* blood-brain barrier system, the same authors found that *C. albicans* cells coated with plasminogen had a 4-fold greater ability to cross that barrier and interpreted this as an indication of the importance of the interaction for infection. These results showed that enolase is a plasminogen receptor in *C. albicans*, although the presence of other receptors could not be excluded.

Phosphoglycerate mutase. Phosphoglycerate mutase, Gpm1, is an enzyme that catalyzes the interconversion of 3-phosphoglycerate and 2-phosphoglycerate and participates in both glycolysis and gluconeogenesis. Using a protein microarray from the yeast *S. cerevisiae* to find proteins able to bind complement regulators, Poltermann et al. (81) identified Gpm1. Since *C. albicans* Gpm1 had an amino acid sequence about 80% similar to that of the *S. cerevisiae* enzyme and was among the plasminogen-binding proteins identified by Crowe et al. (78), the characteristics of this protein and its interactions were studied in detail. Using a specific antiserum against *C. albicans* Gpm1, Poltermann et al. (81) showed by different techniques that the protein was not only present in the cytoplasmic fraction but also on the surface of cells, appearing prominently at the tip of hyphal forms. The Gpm1-plasminogen interaction was inhibited by the lysine analog α-aminocaproic acid, thus implicating lysine-binding domains of plasminogen in this interaction (80). Plasminogen bound to Gpm1 was converted to plasmin when treated with the urokinase-type plasminogen activator, and the plasmin formed could hydrolyze a chromogenic substrate. A strain with a deletion of the *GPM1* gene showed a reduction in plasminogen-binding activity (81), and strains with higher Gpm1 expression levels had greater plasminogen-binding activity (82). Gpm1 has another moonlighting role that will be considered in the next section.

Glycerol-3-phosphate dehydrogenase. Glycerol-3-phosphate dehydrogenase catalyzes a reaction ancillary to the glycolytic pathway, the reduction of dihydroxyacetone phosphate to glycerol-3-phosphate using NADH. In *S. cerevisiae*, there are two isoenzymes, Gpd1 and Gpd2, that appear to work in different physiological situations (83). During a study of *C. albicans* proteins that might be involved in complement evasion, Luo et al. (84) identified the homologue of *S. cerevisiae* Gpd2 as one of them (see the next section). Using a specific antiserum, they showed that Gpd2 was found not only in the cytosol but also at the surface of *C. albicans* in both the yeast and hyphal morphologies. Using an enzyme-linked immunosorbent assay, these authors demonstrated that plasminogen bound to immobilized Gpd2 and that this binding was inhibited by α-aminocaproic acid, a lysine analog, implicating lysine residues of plasminogen in this interaction, as in the cases mentioned before. Plasminogen bound to Gpd2 could be acti-

vated to plasmin by the urokinase-type plasminogen activator, and the plasmin generated was active, cleaving fibrinogen or a synthetic artificial substrate. Like Gpm1, Gpd2 plays another moonlighting role that will be described in the next section.

Enzymes Binding Complement Regulators

The complement system is an important barrier against infective organisms. This system comprises about 30 proteins that participate in a cascade of proteolytic reactions that lead to the assembly of a complex of proteins that form a pore in the pathogen and cause its lysis (85). Several regulators inhibit different reactions in the cascade to avoid attack of host tissues by the proteins of the complement system (86). Among them are factor H (FH) and C4b-binding protein (C4BP), the main fluid-phase inhibitor of the classical and lectin complement pathways (86). Some invading organisms are able to recruit regulators of the complement system of the host and use them to escape from its attack (87). *C. albicans* cells bind different complement regulators (88, 89), and further studies have identified the sugar transporter Hgt1 and the glycolytic enzymes Gpm1 and Gpd2 as blockers of the action of the complement system through their binding to regulators.

Sugar transporter Hgt1. To identify possible proteins of *C. albicans* able to bind the complement regulator FH, Lesiak-Markowicz et al. (90) screened a *C. albicans* expression library by incubation with serum and anti-FH antibodies. Among the positive colonies, a great number contained DNA with a sequence corresponding to the glucose transporter Hgt1 (91). Homozygous and heterozygous *hgt1* mutants were constructed and studied. Using fluorescence microscopy and flow cytometry assays, the authors found that binding of FH to *C. albicans* was much lower in the homozygous *hgt1* mutants than in the parental strain. Reintroduction of the *HGT1* gene, encoding Hgt1, restored the binding to levels comparable to those in wild-type cells. In the same work, Lesiak-Markowicz et al. (90) reported that binding of *C. albicans* cells to another complement regulator, C4BP, was also decreased in the homozygous *hgt1* mutant. To obtain evidence about the *in vivo* significance of those findings, deposition of terminal complement complexes in the wild type, homozygous *hgt1* mutants and mutants with the reintroduced gene *HGT1* was examined. After incubation of the different cells with human serum, the different populations were examined by flow cytometry. In a homozygous *hgt1* mutant, increased deposition of the complement complexes was observed, a result consistent with a role for Hgt1 in blocking complement action.

It is interesting that although there are many genes in *C. albicans* encoding putative glucose transporters, some of them, such as Hgt2, with an amino acid sequence about 90% identical to that of Hgt1 (92), Lesiak-Markowicz et al. (90) found only *HGT1* in their screening. Whether this result indicates a strong specificity for *HGT1* or if it could be due to an incomplete representation of the other genes in the library used remains an open question.

Phosphoglycerate mutase. As stated above, Poltermann et al. (81) observed that the glycolytic protein Gpm1 was also present on the *C. albicans* cell surface. Using an enzyme-linked immunosorbent assay, these authors showed that Gpm1 was able to bind the complement regulators FH and FHL-1 but not the regulator C4BP. Moreover, they showed that the Gpm1-bound factors retained biological activity, as shown by the appearance of degradation products of the protein C3b after adding C3b together with factor I to immobilized Gpm1 with the attached factors. A homozygous *gpm1* mutant showed only a modest reduction in binding to those factors, a result interpreted as indicative of the existence of other binding proteins in the cell. By constructing several deletions in the FHL-1 and FH proteins, Poltermann et al. (81) were able to define the regions of these factors necessary to bind to Gpm1. These proteins contain short consensus repeats (SCRs) of about 60 amino acids (86); while FH uses regions in SCR6 and SCR7 and in SCR19 and SCR20 to bind Gpm1, FHL-1 utilizes only SCR6 and SCR7.

Glycerol-3-phosphate dehydrogenase. In addition to its plasminogen-binding capacity, Gpd2 is able to bind two proteins, FH and FHL-1, that are downregulators of the complement system (84). After a *C. albicans* extract was passed through a column with immobilized FH and the retained proteins were eluted, a protein of 52 kDa in the eluate was identified as Gpd2. Binding of Gpd2 to FH and FHL-1 was confirmed by using procedures similar to those described above for Gpm1, and it was also demonstrated that both FH and FHL-1 bound to Gpd2 were active as complement regulators. Using deletion constructs of FH and FHL-1, the authors concluded that these proteins bind to Gpd2 mainly via SCR7.

Hal3 FROM *SCHIZOSACCHAROMYCES POMBE* IS A MOONLIGHTING PROTEIN AND, IN ADDITION, HAS ANOTHER FUNCTION ORIGINATED BY GENE FUSION

The proteins of *S. cerevisiae* that participate in the decarboxylation of phosphopantothenoyl-L-cysteine to pantotheine-4-phosphate, one of the steps in the biosynthetic pathway of CoA from pantothenate, have been identified in the first decade of the 21st century (93). A heterotrimer formed by the protein Cab3 and two monomers that can be either Hal3 or Vhs3 is required to catalyze that reaction in budding yeast. Since Hal3 and Vhs3 had been identified previously as inhibitors of *S. cerevisiae* serine/threonine phosphatases Ppz1 and Ppz2 (94, 95), they qualify as moonlighting proteins (96). The discovery of the function of these proteins in CoA synthesis was followed by the mapping of the functional domains of *S. cerevisiae* Hal3 (96). Hal3 consists of a core domain of about 250 amino acids with important sequence homology to phosphopantothenoylcysteine decarboxylases (PPCDCs) from other organisms, an N-terminal domain without marked similarity to other proteins and a C-terminal domain rich in acidic residues. While both the N- and C-terminal segments of the protein appear to be important for the inhibition of Ppz1, the core region is essential for Ppz1 binding (96). Some mutations in this region interfered with the inhibitory action on Ppz1 but did not affect the PPCDC activity (95–97), indicating that the distinct functions of the protein do not depend on separate regions of the sequence. Recent results have shown that Hal3 binds Ppz1 as a monomer with a 1:1 stoichiometry and can also readily enter into multimeric complexes, while Vhs3 tends to form stable complexes more resistant to the exchange of monomers with other multimers (98). The authors speculate that the differences in the capacity for oligomer exchange might impinge on the regulation of the moonlighting functions of these proteins.

In an interesting evolutionary study, the same group examined the properties of the corresponding protein in the fission yeast *S. pombe* (99). Although there has been some controversy about the precise location of *S. pombe* on the fungal evolutionary tree (100), it is clear that the ancestors of *S. cerevisiae* and *S. pombe* separated quite early, with estimations ranging between 420 and 330 million

years ago (101). Inspection of the *S. pombe* genome sequence showed only a putative gene (SPAC15E1.04) whose N-terminal region showed about 40% sequence homology to Sc*HAL3*. Surprisingly, this gene also showed a 3′ fragment with sequence homology to thymidylate synthase-encoding genes. The authors resequenced the genomic locus of *S. pombe* and that of the related yeast *Schizosaccharomyces japonicus* and confirmed the unusual structure of the gene in both cases (99). The same authors also showed that the *S. pombe* Hal3 protein or even its N-terminal domain could rescue an *S. cerevisiae cab3* single mutant or *hal3 vhs3* double mutant, indicating that this protein is sufficient to achieve PPCDC activity. The protein was also able to inhibit the *in vitro* activity of *S. cerevisiae* Ppz1 or the corresponding *S. pombe* homolog Pzh1. In addition, Hal3 from *S. pombe* or its C-terminal domain allowed the growth of a *cdc21* mutant of *S. cerevisiae* (99) whose phenotype is due to a lack of thymidylate synthase. Thus, Hal3 from *S. pombe* has moonlighting activity and is also a "bifunctional" enzyme that acquired a completely unrelated activity, probably by gene fusion; according to Molero et al. (99), such fusion events are rare in *S. pombe*, and only 12 were found by using a dedicated algorithm.

What is the situation of PPCDCs in other yeasts? Exploration of genomic sequences within the hemiascomycetaceous yeasts suggests the existence of heterotrimeric PPCDCs with two variants. In the yeast group derived from an ancestor with a duplicated genome (102, 103), to which *S. cerevisiae* belongs, the enzyme would be formed by one Cab3 subunit plus two units of Hal3 and/or Vhs3. In archiascomycetaceous yeasts, to which *S. pombe* belongs, a homotrimeric protein formed by a Hal3-like protein is found (93). This diversity is conditioned by the structure of the active enzyme. PPCDCs are trimers in which the catalytic sites form at the interfaces between the different subunits. Specific cysteine, histidine, and asparagine residues are required for catalysis. These residues are provided by a single protein in homotrimeric PPCDCs. In *S. cerevisiae*, the functional histidine residue is provided by Hal3 or Vhs3 and the cysteine and asparagine residues are provided by Cab3, while in other yeasts, the residues are provided by Cab3 and Hal3- or Vhs3-like proteins. A protein with PPCDC activity similar to that of the N-terminal domain of *Sp*Hal3 could have been the primitive structure from which the others originated. This original form may have already possessed the moonlighting capacity.

FINAL REMARKS AND PERSPECTIVES

In this article, we have considered the experimental evidence that qualifies as moonlighting proteins a number of yeast proteins that were not identified as such in previous reviews. Their disparate canonical functions and the wide range of moonlighting activities are astonishing and show the power of evolution to produce new functions from extant materials. The biochemical and genetic potentialities of yeasts emphasize their importance in studying moonlighting functions, not only of yeast proteins but also of proteins from other organisms, as shown by Simonicova et al. (104), who used *S. cerevisiae* to study extratelomeric functions of mammalian telomerase.

Mutational Analysis and Structural Studies

Results showing the participation of a protein in two dissimilar functions might be a promising hint at a possible moonlighting protein. However, this is not always the case, as the noncanonical activity may be a secondary effect from a metabolic defect. An example of this is fumarase, a metabolic enzyme from the tricarboxylic acid cycle, whose absence may increase intracellular levels of fumarate, making yeast cells more sensitive to stresses producing double-strand breaks in DNA (105, 106). Another example is the participation of Sod1 in glucose repression of respiration in yeast, which depends on the catalytic activity of the protein (44).

An unequivocal identification of a protein as moonlighting would ideally require mutational studies. Several types of mutation could be expected in that type of protein, some affecting both the canonical and moonlighting functions, others located in the catalytic region and affecting only the canonical activity, and still others affecting only the moonlighting region and yielding a metabolically unaltered phenotype. Examples of the different types of mutations are found for yeast hexokinase (107), galactokinase (108), and some other yeast proteins (18), as well as for mammalian aldolase (109). It has been reported recently that a glyceraldehyde-3-phosphodehydrogenase may play a moonlighting role in *S. cerevisiae* (110). The isoenzyme Tdh3 has been shown to interact with the sirtuin Sir2 and to facilitate transcriptional silencing at the telomeres. In this work, a number of *tdh3* mutants have been used and it has been observed that there is no correlation between glyceraldehyde-3-phosphodehydrogenase activity and the degree of silencing at a telomere. This is a strong indication that the moonlighting effect is not a side effect of the metabolic activity.

In some clear cases of moonlighting, such as the pyruvate carboxylase of *H. polymorpha*, only mutant proteins that have lost their moonlighting activity have been obtained (19). Mutational analysis of the Tps1 (trehalose-6-phosphate synthase) protein of *S. cerevisiae* suggests that it may be a moonlighting protein. This protein produces a regulator of glycolysis (111, 112) and appears to be implicated, independently of its metabolic activity, in resistance to different stresses (113). Reinforcing its possible moonlighting qualification is the finding that, in the rice blast fungus *Magnaporthe grisea* (now *oryzae*), the Tps1 protein itself, and not its catalytic activity, is required for efficient plant infection (114).

Mutational studies would be critical to ascertain if the moonlighting umbrella could apply to all proteins that, although usually cytosolic, are also found extracellularly and act in this form to facilitate invasion of the host by pathogenic organisms (13, 115). A detailed structural study has been made for the surface enolase of *Streptococcus pyogenes* and *S. pneumoniae*. Enolase is a glycolytic enzyme found to be able to bind to plasminogen. Derbise et al. (116) constructed different mutant forms of the enolase from *S. pyogenes* affected in the C-terminal region that retained normal enzymatic activity but exhibited decreased plasminogen-binding activity. Cork et al. (117) obtained a series of mutations located on the surface of the octameric protein of *S. pneumoniae*; some of them conserved activity but had lost the capacity to bind to plasminogen. Mutant proteins binding plasminogen but devoid of catalytic activity were not isolated.

A puzzling issue regarding proteins occasionally found extracellularly is how they reach the cell surface in the absence of a conspicuous secretion signal. Different structural characteristics appear to be involved in the process, as demonstrated by the work on the glyceraldehyde-3-phosphate dehydrogenase of *S. pyogenes* and on the enolase of *Bacillus subtilis*. Addition of a C-terminal hydrophobic tail to the glyceraldehyde-3-phosphate dehydrogenase retained it in the cytosol without affecting its enzymatic activity and the strain carrying it lost many of its adhering activities (118).

Deletion of an α-helix stretch from the terminal region of the enolase resulted in a protein that was not exported; replacement of that stretch with a segment with α-helix structure from β-galactosidase restored the exportation of the protein (119).

Databases and Prediction of Moonlighting Functions

An important development since the appearance of previous reviews has been the increased use of powerful informatic tools in the moonlighting field. This could change the way in which moonlighting proteins are discovered. The development of databases specifically devoted to moonlighting proteins will increase the accessibility of data on such proteins and facilitate the tracking of new additions to this category. Two such databases have recently been made available, Moonprot (5) and MultitaskProtDB (17). When it appeared, the first one, searchable at http://www.moonlightingproteins.org, presented information on more than 200 moonlighting proteins experimentally verified to behave as such; at its publication, the second one, accessible at http://wallace.uab.es/multitask, offered different information on about 300 multitasking proteins. Critical for the usefulness of databases is the quality of annotations; care should be taken to include in them only well-authenticated cases of moonlighting, complying with the criteria defining this type of protein. Discrimination among moonlighting, pleiotropy, and secondary metabolic effects is crucial to avoid the incorporation of inadequate proteins to the databases.

Prediction of moonlighting activity is an outstanding pending task. Homology comparison is not enough, because if the moonlighting function depends on small structural changes, the global identity score may be uninformative. Attempts at and advances in moonlighting prediction have been reviewed by Khan and Kihara (120). Using Gene Ontology annotations, protein-protein interaction networks, and other omics data, Khan et al. (121) were able to predict moonlighting functions for several *E. coli* proteins, a prediction confirmed by results in the literature. Chapple et al. (122) used protein-protein interaction networks to predict proteins with multiple functions. They coined the term "extreme multifunctional proteins" for them and proposed that moonlighting proteins could be seen as a subset of the extreme multifunctional protein set. Using protein network information, together with current protein annotations, they identified 430 proteins in the human interactome as candidates for extreme multifunctional proteins. Among them, they looked for 39 known moonlighting proteins and found only 6. They attributed this low score to a lack of adequate information in the annotations. However, an alternative explanation could be that many moonlighting proteins do not interact with multiple proteins and therefore would not form part of the set of extreme multifunctional proteins.

Evolution of Moonlighting Proteins

The question of how moonlighting functions arose in the course of evolution is a matter of speculation, although some hints may be advanced. Because the canonical activity of many proteins is conserved between related yeast species but moonlighting activities are not, it may be assumed that they have appeared at different moments during evolution. Also, the fact that a multitude of similar enzymes perform similar reactions in disparate organisms while the moonlighting activities of the corresponding enzymes are observed in only some of them seems consistent with the previous idea.

Moonlighting proteins belong to a category different from that of pseudoenzymes, proteins whose sequences are almost identical to those of active proteins but that do not catalyze the corresponding chemical reactions; they have been shown to have regulatory functions in a number of cases (123–125). It appears likely that pseudoenzymes evolved from a protein with enzymatic activity and then lost this activity while acquiring a regulatory capacity. Nevertheless, it may be conjectured that in some cases the ancestor of a pseudoenzyme might have been a moonlighting protein that lost its enzymatic activity and conserved a regulatory function. It is important to consider that the acquisition of an additional function by a protein requires a tradeoff between improvement of the new function and conservation of the existing function. The appearance of a new function may take advantage of structural features involved in the canonical reaction. A possible example is the Pet54 protein of *S. cerevisiae*. The lack of Pet54 affects both the translation of the *COX3* mRNA and the splicing of an intron from the *COX1* pre-mRNA, each function requiring a different protein domain within Pet54 (126). Kaspar et al. (127) have shown that Pet54 is able to bind to regions in the 5′ untranslated leader of the *COX3* mRNA and in the intron of *COX1* pre-mRNA which show 56% sequence similarity, and that the binding involves the same surfaces of Pet54. These authors suggested that the original functions of Pet54 were to bind *COX3* mRNA and to recruit Pet122 and Pet494 to form a complex to activate mRNA translation. Later on, the capacity of Pet54 to bind the intron region of *COX1* pre-mRNA may have allowed the selection of variants of Pet54 able to participate in intron splicing.

In some cases, changes in the functional properties of a protein need to follow what may appear to be a tortuous trajectory, as shown by Harms and Thornton (128) in a study of the evolution of glucocorticoid receptor specificity. They discussed how, for an ancestral glucocorticoid receptor to acquire specificity for cortisol, several modifications of the protein that did not appear to modify its function were necessary before the protein could tolerate the mutations that conferred cortisol specificity. Those mutations were needed to stabilize the protein scaffold to allow the ulterior structural changes.

It should also be taken into account that the appearance of a moonlighting function may be influenced by the existence, and eventual evolution, of a partner with which to interact. An example may be the moonlighting function of pyruvate carboxylase in the assembly of methanol oxidase (129). Only pyruvate carboxylases from methylotrophic yeasts exhibit this capacity, while the enzymes from nonmethylotrophic yeasts, in spite of their great sequence similarity, lack this ability. This is probably due to the lack of selective pressure in the absence of a partner with which to interact during their evolutionary history.

Experiments to generate proteins with moonlighting properties and to infer from them the original evolutionary pathway of the naturally evolved proteins may provide insights into the regions of the protein important for the appearance of the moonlighting function (130). Nevertheless, it could be problematic to infer from results of this type of experiment the original evolutionary pathway of the naturally evolved protein. As Koschwanez et al. (131) have pointed out, results from an engineering strategy might not reflect the evolutionary pathway exactly, as other constraints may have been at work that are not mimicked in the engineered process. But even with this caveat, that type of experiment could provide hints about possible evolutionary events leading to the ap-

pearance of moonlighting functions. Jeffery (20) has pointed out that information derived from such experiments may also be useful in the design of proteins with new functions.

Some Unanswered General Questions

There are several general questions about moonlighting, not specific to yeasts, that are worth considering. One of them is how moonlighting proteins reach their partners and how they discriminate among the different tasks. Interactions of the proteins will be controlled by the intrinsic properties of the corresponding proteins and by changes in the metabolite landscape that determine their actual configuration. Modifications in folding in response to a ligand might provide opportunities to show new properties. An illustrative example of this has been provided by Ha et al. (132). They constructed a chimeric protein resulting from the fusion of a barnase and the Gcn4 DNA-binding domain in a way that prevents the fragments from being simultaneously folded and active. If a specific fragment of DNA is bound to the Gcn4 part, barnase unfolds and becomes inactive, while in the absence of DNA, the barnase domain is folded and active.

In a certain number of cases, moonlighting functions require movement of the protein to another cellular compartment. The moonlighting action of several proteins, such as Lys20, Sod1, and Zuo1, takes place in the nucleus. The increasing number of mitochondrial proteins also found in the nucleus suggests that some of them may also have moonlighting roles and participate in regulatory circuits (133). The translocation process of moonlighting proteins has not been studied in detail, except in the case of hexokinase 2, a metabolic enzyme that moonlights as a transcriptional repressor (22). Moreno and Herrero have discovered that the localization of the protein is determined by the phosphorylation state of a serine residue that, in turn, depends on the activities of the protein kinase Snf1 and the protein phosphatase Glc7 (134). It has also been reported that the phosphorylation of that serine residue is blocked in a mutant devoid of the protein kinase Tda1 (135). In addition, the traffic of hexokinase 2 between the cytoplasm and the nucleus implies the importins Kap60/Kap95 and the exportin Xpo1 (Crm1) (134).

Moonlighting action may or may not affect the canonical activity of the protein; binding of galactokinase to the inhibitory protein Gal80 blocks galactokinase activity (136), but binding of arginase to ornithine transcarbamylase does not abolish arginase activity (137).

CONCLUSIONS

Since the moonlighting protein concept was established, realization of its importance has been slowly but steadily permeating the general biochemical knowledge. As moonlighting proteins participate in diverse cellular functions and receive signals from molecules involved in different pathways, they might provide cross talk between different cellular processes, facilitating the cells' response to changes in their environment. In spite of the advances in the knowledge of moonlighting proteins, important gaps remain to be filled to understand their intimate action mechanisms and their regulation.

The implication of moonlighting proteins from pathogenic bacteria in invasive processes has added a new dimension to this class of proteins, and the finding that some pathogenic yeasts might use moonlighting proteins to help invasion extends these observations.

The different processes in which moonlighting proteins participate, not only in yeasts but also in other organisms, including higher eukaryotes, concern fields as diverse as medicine or biotechnology, making it important to widen and deepen the current knowledge about these molecules and their modes of action.

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