Mass Spectrometry of Ribosomes from *Saccharomyces cerevisiae*

IMPLICATIONS FOR ASSEMBLY OF THE STALK COMPLEX*

The acidic ribosomal P-proteins form a distinct protuberance on the 60 S subunit of eukaryotic ribosomes. In yeast this structure is composed of two heterodimers (P1α-P2β and P1β-P2α) attached to the ribosome via P0. Although for prokaryotic ribosomes the isolation of a pentameric stalk complex comprising the analogous proteins is well established, its observation has not been reported for eukaryotic ribosomes. We used mass spectrometry to examine the composition of the stalk proteins on ribosomes from *Saccharomyces cerevisiae*. The resulting mass spectra reveal a noncovalent complex of mass 77,291 ± 7 Da assigned to the pentameric stalk. Tandem mass spectrometry confirms this assignment and is consistent with the location of the P2 proteins on the periphery of the stalk complex, shielding the P1 proteins, which in turn interact with P0. No other oligomers are observed, confirming the specificity of the pentameric complex. At lower m/z values the spectra are dominated by individual proteins, largely from the stalk complex, giving rise to many overlapping peaks. To define the composition of the stalk proteins in detail we compared spectra of ribosomes from strains in which genes encoding either or both of the interacting stalk proteins P1α or P2β are deleted. This enables us to define novel post-translational modifications at very low levels, including a population of P2α molecules with both phosphorylation and trimethylation. The deletion mutants also reveal interactions within the heterodimers, specifically that the absence of P1α or P2β destabilizes binding of the partner protein on the ribosome. This implies that assembly of the stalk complex is not governed solely by interactions with P0 but is a cooperative process involving binding to partner proteins for additional stability on the ribosome.

Ribosomes are the universal translators of messenger RNA into proteins. X-ray analysis of the 30 S subunit from *Thermus thermophilus*, the 50 S subunit from *Halococcus marismortui*, and the intact 70 S particle from *T. thermophilus* have revealed fascinating insights into the structures of these macromolecular machines (1–3). There are currently, however, no high resolution structures of eukaryotic ribosomes. These 80 S particles consist of two subunits, the 60 and 40 S subunits. Low resolution structures obtained by electron microscopy indicate an overall topology similar to that of the *Escherichia coli* 70 S ribosome but indicate that the eukaryotic particle is larger and more complex. In general, the core of the ribosome is highly conserved, but in *S. cerevisiae* there are large differences in the periphery of the particle. This is attributed to the presence of additional ribosomal proteins and ribosomal RNA (rRNA) regions of variable size, interrupting the universal core secondary structure of eukaryotic rRNA (4). The eukaryotic 60 S subunit is larger than the prokaryotic 50 S subunit, but the greatest differences are observed between the 30 S subunit and the eukaryotic 40 S subunit. A common feature of all ribosomes is a flexible protuberance on the side of the large subunit, known as the stalk complex. The presence of the stalk has been reported for all species examined to date but is often difficult to study by x-ray analysis because of its dynamic nature. The pentameric complex from bacterial sources however has been studied in some detail in complex with ribosomes by cryo-electron microscopy to determine conformational changes associated with ribosome function (5). The stoichiometry of the stalk complex in *E. coli* has been determined to be two copies of L12, with along its acetylated counterpart L7, and one copy of L10 at the base of the stalk. In higher organisms the stalk has evolved from two L7/L12 dimers to a tetramer of acidic "P-proteins." In ribosomes from *S. cerevisiae* there are five stalk proteins: P0, P1α, P1β, P2α, and P2β. Despite some similarities to L7/L12, sequence identity between prokaryotic and eukaryotic acidic ribosomal proteins is low and is restricted mainly to the hinge region between the N- and C-terminal domains. The P1 and P2 proteins vary in size between 10.6 and 11.1 kDa and are structurally and functionally distinct (6), although they share a highly conserved C terminus. The P1 proteins share a conserved N terminus, as do the P2 proteins. No post-translational modifications of the P-proteins have been reported other than a single phosphorylation site located at serine 96 in P1α, P1β, and P2α and position 100 in P2β (7). Phosphorylation is not believed to be a requirement for their association with the ribosomal particle (7). It has also been proposed, however, that phosphorylation is involved in degradation of P1 proteins and that it may affect expression of certain proteins by altering the translation of specific mRNAs (8, 9).

The stability in solution of the stalk complex from yeast is established as significantly lower than that of its bacterial counterparts (10) and consequently has not been isolated as an intact species. It is also established that the stalk proteins from yeast are capable of exchanging with free proteins in the cytoplasmic pool (11). The stalk complex is associated with the *S. cerevisiae* ribosomes via interactions of P0 with L12. L12 is functionally equivalent to L11 from *E. coli* and binds to the

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same highly conserved rRNA region as L11 in \( E.\ coli \), located immediately below the P0 binding site via a highly conserved rRNA binding domain. P0, the eu- karyotic equivalent of bacterial L10, binds through its highly conserved C-terminus to the stalk proteins (12), anchoring them to the ribosome. P0 has the same 11-amino acid C-terminal sequence as P1 and P2, and the phosphorylation site, Ser-902 in P0, is within this conserved C-terminus. Examination of the stalk assembly both in vitro and in \( \textit{vitro} \) has revealed that P2β is unable to bind to the ribosome in the absence of P1α; in contrast P1α is capable of binding to the particle in the absence of P2β (13). These results have been interpreted to suggest that assembly of the stalk complex is a coordinated process in which P1 proteins provide anchorage to the ribosome for the P2 proteins, whereas the P2 proteins confer functionality to the complex (13). Recent evidence has also shown that the heterodimer P1α-P2β is a stable entity in solution; this observation led to the proposal that the P proteins form dimers in the cytoplasm prior to assembly on the ribosome (14). However, the precise order of events in the assembly of the stalk complex remains unclear.

Previously we applied nanoflow electrospray ionization mass spectrometry to study intact \( E.\ coli \) ribosomes and showed that it is possible to maintain the noncovalent interactions between the three large RNA molecules and 54 proteins in the gas phase (15, 16). Moreover, we established a correlation between the surface area of interaction of 50 S ribosomal proteins with ribosomal RNA and their propensity to dissociate in the gas phase of the mass spectrometer. Consistent with this finding, the most prominent peaks in the mass spectra of \( E.\ coli \) ribosomes are assigned to proteins from the stalk region, which do not interact directly with RNA. Given this fact, the primary aim of the present study is to investigate the interactions in the stalk regions of ribosomes from wild type and variant strains of \( S.\ cerevisiae \). We investigated the effect on the stalk complex of deletion of one or more P1/P2 proteins by introducing into the mass spectrometer ribosomes from three \( S.\ cerevisiae \) strains that do not have a full complement of P1 and P2 proteins: D5, in which P2β is absent, D7, in which P1α is absent; and D57, in which both P2β and P1α are absent. The results reveal post-translational modifications and have implications for assembly of the stalk complex.

**EXPERIMENTAL PROCEDURES**

**Preparation of Yeast Ribosomes—**\( S.\ cerevisiae \) ribosomes were prepared according to the method described previously (17). Briefly, the \( S.\ cerevisiae \) strains used were wild type strain W303-1b and strains D5 (P2β absent), D7 (P1α absent), and D57 (P2β and P1α absent). D57 cells were produced by crossing D5 and D7 haploid strains followed by selection and isolation of colonies harboring two genetic markers (18). Yeast cells were grown to midlog phase in YEPD (1% yeast extract, 2% peptone, 2% glucose). \( E.\ coli \) strain DH5α was grown in Luria Broth medium (1.0% bacto-tryptone, 0.5% yeast extract, 0.5% NaCl) and used for transformation and propagation of plasmids. The cells were broken with glass beads, and the extracts were centrifuged for 15 min at 15,000 rpm. Ribosomes were prepared from high speed centrifugation of the pellet at 100,000 \( \times \) g and were washed by centrifugation for 20 h at 45,000 rpm through a 20–40% sucrose gradient in 30 mM Tris-HCl, pH 7.4, 50 mM MgCl\(_2\), 500 mM NH\(_4\)Cl, and 5 mM Mg-mercaptoethanol. 50 mM MgCl\(_2\) in the sucrose gradient was used to compensate for the high MgCl\(_2\) in the sucrose gradient was used to compensate for the high concentration of ammonium ions and was found to be optimal for removing nonribosomal proteins from the particles. The ribosomes were recovered in the pellet and were resuspended in 20 mM Tris-HCl, pH 7.4, 12.5 mM MgCl\(_2\), 80 mM KCl, and 5 mM Mg-mercaptoethanol (Buffer 1).

**Isoelectric Focusing—**Ribosomes (500 \( \mu \)g) in \(~25\) ml of Buffer 1 were treated with RNase (10 \( \mu \)g/\( \mu \)l of ribosomes) for about 1 h on ice. The sample was then neutralized, dissolved in 20 ml of loading buffer (6% amphotolys, 8 M urea), and resolved by vertical 5% polyacrylamide isolectric focusing in a 2.0–5.0 pH range as described previously (8).

**Western Blotting—**Western blotting was performed after proteins were separated on 15% SDS-PAGE and blotted onto polyvinylidene

**Phosphatase Treatment—**Ribosomes (500 \( \mu \)g) were incubated for 1 h at 37°C with 10 units of alkaline phosphatase (New England Enzyme) in 100 ml of SucReCut buffer supplied by the manufacturer. The sample was then supplemented with RNase A and processed as described previously for isoelectric focusing. Ribosomes treated similarly but in the absence of alkaline phosphatase were used as a control.

**Mass Spectrometry—**For mass spectra 1 \( \mu \)l stock solutions (50 \( \mu \)l) were buffer-exchanged into 50 \( \mu \)l of 10 mM ammonium acetate using Bio-spin 6 chromatography columns (Bio-Rad) with a molecular mass cut-off at 6000 Da, equilibrated previously with 10 mM ammonium acetate at pH 7.0. Nanoflow capillaries were prepared as described previously (21). Spectra were recorded on modified Micromass LCT and QToF2 mass spectrometers (22) (Micromass UK Ltd., Manchester, UK). The capillary and cone voltages were maintained at 1800 and 80 volts, respectively, to observe in-source dissociation products from the ribosome. Pressure conditions were maintained in the LCT at 9 \( \times \) 10\(^{-9}\) mbar in the analyzer, 3.2 \( \times \) 10\(^{-7}\) mbar in the ToF and 6 \( \times \) 10\(^{-7}\) mbar in the QToF2. Tandem mass spectra were recorded on the QToF2 with a collision cell voltage of 150 V and a collision gas of argon at a pressure of 3.5 \( \times \) 10\(^{-8}\) mbar.

Analysis of mass spectra was performed with Masslynx, version 3.1 (Micromass UK Ltd.). The average masses of protein species were calculated from at least three charge states. All spectra were calibrated externally against spectra recorded for cesium iodide (100 mg/ml). The masses measured are centroided values. All mass spectra underwent minimal smoothing with no resolution enhancement and are the average of 5–20 2-s scans.

**RESULTS**

Solutions of ribosomes from wild type \( S.\ cerevisiae \) at pH 7.0 in 10 mM ammonium acetate were introduced into the mass spectrometer, and spectra were recorded under conditions selected to induce in-source dissociation of proteins and complexes from the intact ribosome (see “Experimental Procedures”). A typical mass spectrum is shown in Fig. 1A. At high \( m/z \) values (above 4000 \( m/z \)), a series of broad peaks were observed corresponding to a species with a mass of 77,291 \( \pm \) 7 Da. This mass is higher than that of any of the reported ribosomal proteins (23), implying that it arises from association of proteins in a noncovalent complex. By analogy with our previous studies of bacterial ribosomes, the most likely origin of these peaks is from the dissociation of the intact stalk complex (15, 16). This mass is consistent with the overall stoichiometry P10, P16, P26, P2β, and P0 (calculated mass of the unmodified pentamer, 77,154 Da). However it is not possible to confirm the precise composition of this complex from mass measurement alone because of extensive post-translational modifications of the P proteins (see below). To investigate the protein composition of the peaks assigned to the stalk complex, we therefore carried out a tandem mass spectrometry experiment to determine the dissociation products from a single charge state (19–). Activation of this complex yields the spectrum shown in Fig. 1B.

For tandem mass spectrometry of multiprotein complexes, a universal mechanism for dissociation is emerging in which individual proteins or small molecules are released from the isolated complex to leave “striped complexes” at higher \( m/z \) (24). At the high \( m/z \) region of this spectrum, close to the peak that was isolated, a peak was assigned to a charge state of the pentameric complex (18+) arising from loss of positive charge (Fig. 1B) (presumably ammonium ions from the buffer employed in the introduction of the ribosome solution). A second series of ions can also be discerned, with the mass of the predominant peak consistent with the stalk complex with loss of P2β. The width of these high \( m/z \) peaks is also consistent with the loss of other P proteins, although the predominant series can be assigned to loss of P2β. At the low \( m/z \) region of
the spectrum, Fig. 1C, the four individual P1 and P2 proteins are observed, consistent with their release from the intact stalk complex. Previous studies (25) have shown that the extent to which individual proteins are released in tandem mass spectrometry is related to their location in the complex, such that those on the periphery of the complex are released prior to those located at the center. It is interesting to note that the intensity of the peaks assigned to the P2 proteins is greater than for the P1 proteins.

It has been proposed that the P1 proteins interact strongly with P0 and that P2 proteins are associated with the stalk predominantly through interactions with P1 (see schematic representation of the interactions of the stalk proteins in Fig. 1) (17). Consequently, P2 proteins are released in preference to the P1 proteins, consistent with the location of P2 proteins on the periphery of the complex. Moreover, P0 is not observed in this spectrum in accord with its central position and number of interactions with the P1 proteins. These observations allow us to conclude that the charge state that was isolated from the 77-kDa complex contains all four of the P1/P2 proteins. The difference in mass between the sum of the four P1/P2 proteins and the pentameric stalk is consistent with the presence of P0. Although it is not possible from these experiments to rule out the possibility of different combinations of P1/P2 proteins co-existing with the same stalk complex, recent data are consistent with homogeneous populations of ribosomes carrying a single copy of each of the P proteins (27). Taken together these results therefore establish that a population of ribosomes with the pentameric stalk complex is present in solution and demonstrate that the interactions between these proteins can be maintained in the mass spectrometer, despite their reduced stability when compared with their prokaryotic counterparts.

At low m/z values the mass spectrum contains numerous peaks that can be assigned on the basis of mass to ribosomal proteins, with the most prominent in the spectra assigned to the acidic stalk proteins (Fig. 1A). In addition to these multiplet peaks, proteins P0 and L12 at the base of the stalk are readily observed. A minor series of peaks in the spectrum results from dissociation of the large subunit protein L1. Expansion of the low m/z region of the spectrum (Fig. 2) reveals in excess of 30 peaks over only ~100 m/z units. The complexity in this spectrum derives from multiple metal ions binding to the proteins that in addition have a number of post-translational modifications. A common observation for many of the neighboring peaks is that they are separated by a mass of 22 Da. This could be due to either sodium adducts, often present in electrospray mass spectra, or to binding of divalent magnesium ions. Sodium and magnesium ions replace one and two protons, respectively, giving rise to identical unit mass differences of 22 Da in mass spectra. Since magnesium is present in the storage buffer to maintain associations between ribosomal subunits, and given that this is removed by the addition of chelating
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![Figure 2](image)

Fig. 2. Expansion from *m/z* 1760 to 1885 revealing the complexity of the +6 charge states of the ribosomes from wild type, **P1α/β**, and **P2α/β** and additional proteins **L1 (+13)** and **P0 (+18)**. Based on measurement of the masses, the peaks are assigned to modifications including phosphorylation, acetylation, trimethylation, and multiple magnesium adducts. However, substantial overlap of the peaks prevents precise assignment of these peaks. The peaks are labeled as the trimethylated form of the protein but cannot be distinguished from the acetylated forms.

agents such as EDTA (data not shown), we conclude that the metal binding to the acidic proteins in *S. cerevisiae* ribosomes is magnesium.

It is also important to note that the mass of two Mg\(^{2+}\) ions (44 Da) binding to the protein is not readily distinguished from the possibility of acetylation (42 Da) or trimethylation (42 Da), both established as post-translational modifications on other yeast ribosomal proteins (7). The distribution of adduct peaks assigned to the proteins, however, can enable the distinction to be made between potential post-translational modification and metal ion binding. For example in the case of the protein **P2α**, the peak 42 Da higher than the unmodified form could be assigned either to acetylation/trimethylation or to binding of two Mg\(^{2+}\) ions. However, this peak is significantly more intense than neighboring peaks assigned to binding of one and three Mg\(^{2+}\) ions. Such an increase in intensity of a single peak is indicative of a post-translational modification, in this case either acetylation or trimethylation. It is established from previous studies, however, that acetylation of the N terminus occurs only for **P1** proteins (28) and has not been reported for **P2** proteins. Based on all the above considerations the identity of the +42-Da peak of **P2α** is either the trimethylated form of the protein or, alternatively, contains an acetylation of an internal lysine residue. Similarly, **L1** has only one additional peak (42 Da) and does not show other peaks that could be assigned to metal binding. This modification is therefore assigned to acetylation, consistent with previous studies (23). Precise definition of the post-translational modifications of the P proteins of ribosomes from wild type yeast is, however, extremely difficult because of the extensive overlap of the peaks and the overall complexity of the spectra. For example, the difference in mass between **P1β** and **P2α** is 78, such that **P2α** cannot be distinguished from **P1β** containing a phosphorylation or a combination of acetylation and magnesium ion binding. Similarly, modifications of **P2α** overlap with the peaks assigned to **P1α**. To overcome this problem and to examine the effects on the stability and interactions of the stalk proteins, we have recorded spectra for ribosomes deficient in specific **P1** and **P2** proteins.

The mass spectrum recorded for yeast strain D57 (in which **P1α** and **P2β** are absent) is shown in Fig. 3B together with that of the wild type recorded under the same conditions for comparison (shown in Fig. 3A). It is apparent that by introducing ribosomes without the full complement of P proteins the complexity of the spectra is reduced. It is also apparent that compared with the spectra recorded for the stalk complex under tandem mass spectrometry conditions (Fig. 1C), many more proteins are released in accord with the high internal energy imparted to ribosomes under these in-source dissociation conditions. **P2α** and **P1β** proteins, **P0** and **L12**, dissociate in the gas phase and form the predominant series in the spectra. An expansion of this spectrum (Fig. 4B) reveals an additional peak 80 Da higher than that assigned to **P2α**, consistent with phosphorylation. Also apparent is a peak assigned to trimethylation of phosphorylated **P2α**. This modified form of **P2α-PO3** is the major species present; very little nonphosphorylated protein is observed. There is no evidence of phosphorylation of **P1β**.

Mass spectra recorded for ribosomes from strains lacking **P1α** (D7) and **P2β** (D5) (Fig. 3, C and D) and in expanded form (Fig. 4, C and D), respectively, were also compared with spectra recorded for wild type (Figs. 3A and 4A). The same post-translational modifications identified in the spectra of D57 are observed, including the phosphorylation and trimethylation of **P2α**. Comparison of the ratio of the modified forms of the
proteins between the various D strains reveals closely similar relative intensities. There is, however, some variation in intensity when comparing the spectra of ribosomes from the D strains with the wild type. It should be noted, however, that this variation cannot be interpreted in terms of absolute quantities of the various proteins but rather is attributed to the ease with which the proteins dissociate from the various ribosomes.

Surprisingly, however, the proteins P1α and P2β are not observed in spectra recorded for ribosomes from any of the three D strains. This was unexpected since in strain D5 the gene expressing P1α has not been deleted and in D7 P2β is expressed. It is also noteworthy that the peaks assigned to the stalk complex, observed for ribosomes from the wild type strain, could not be observed for ribosome solutions from the
various D stains under any mass spectrometry conditions, emphasizing the importance of the full complement of P proteins for the stability of the stalk pentamer.

We investigated the apparent absence of P1 and P2 in strains D5 and D7, respectively, using isoelectric focusing (IEF)\(^1\) (Fig. 5A). We compared strain D7 with wild type and found the expected band for P2 and the absence of a band assigned to P1. Similarly, IEF was used to confirm the presence of P1 but not P2 in strain D5 (data not shown). The question arises as to why these proteins are not detected in the mass spectra of the D strains. A possible explanation is that during buffer exchange protocols used prior to mass spectrometry analysis, P1 and P2 in the absence of their binding partner are absorbed on the filtration device. To investigate this possibility, ribosomes from both the wild type and the D7 strain were subjected to the identical sample preparation procedure used prior to the mass spectrometry, and the IEF analysis was repeated (Fig. 5B). The results show that whereas the proteins in the wild type species can be readily identified, only P1 and P2 can be discerned for ribosomes from strain D7, although the intensity of the P2 is significantly reduced when compared with those from the wild type. These results therefore demonstrate that substantial losses occur during filtration for the partner protein in the absence of its binding partner and are consistent with the mass spectrometry experiments shown in Fig. 4.

Interestingly, of the four different forms of P2 assigned in the mass spectra (unmodified form, phosphorylated, trimethylated, and both trimethylated and phosphorylated species), only unmodified P2 could be readily observed in the silver-stained gel. However, IEF and analysis using a Western blot, employing an anti-P2 monoclonal antibody, revealed the presence of the expected four bands for P2. Treatment with alkaline phosphatase demonstrates removal of the two bands assigned to phosphoforms, whereas the proposed trimethylation remains as expected (Fig. 5C). This demonstrates the sensitivity of this mass spectrometry approach to identification of modified full-length proteins.

In addition to differences in the acidic P proteins, a number of interesting features were observed for other proteins that dissociate from the ribosome in the various strains. Two series of peaks were observed in the mass spectrum of ribosomes from the D strains that are not observed for ribosomes from the wild type (Fig. 3). The masses of these series correspond to two large subunit proteins, L30 and L27 (Table I). Also of interest is the change in the charge state distribution of protein P0. In the mass spectrum recorded for the wild type protein, the charge state series extends from +14 to +18. By contrast, ribosomes from the D strains exhibit charge states for P0 that extend from approximately +26 to +31 as well as an absence of the lower charge states (+14 to +18). A high degree of charging of a protein in an electrospray ionization mass spectrum indicates that the protein has a large number of sites accessible to protonation (29). This would imply that the absence of P1 and P2, which bind directly to P0, results from either a less compact structure of the latter or exposes additional sites for protonation that are shielded by interacting proteins when the full complement of P1/P2 proteins is present.

**DISCUSSION**

The results presented here have probed the effect of deletion of proteins from the stalk complex on the dissociation of proteins from intact ribosomes. Most notable is the absence in mass spectra of the P1 and P2 proteins in the single gene deletion mutants. Also of interest is the existence of a population of P2 with both phosphorylation and trimethylation re-

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\(^1\) The abbreviation used is: IEF, isoelectric focusing.
Isoelectric focusing analysis of proteins from wild type and the various deletion mutants. A, silver-stained gel of wild type W303 and deletion mutant D7 showing the presence of the full complement of P1/P2 proteins for wild type and the absence of P1α in the case of ribosomes from strain D7. B, silver-stained gel of wild type W303 and deletion mutant D7 after the buffer exchange protocol employed for sample preparation prior to mass spectrometry. The absence of the P1α protein and reduction in intensity of P2α is attributed to absorption of these acidic proteins on the filtration device. C, Western blot of ribosomes from wild type strain W303 before phosphatase treatment (left lane) and after phosphatase treatment (right lane) using an anti-P2α antibody. Before phosphatase treatment, four bands are assigned to unmodified P2α, P2β with one phosphorylation site (P2αp), trimethylated P2α (P2αpTM), and trimethylated phosphorylated P2α (P2αpTM). After phosphatase treatment, both P2αp and P2αpTM are absent, and only unmodified P2α and P2αTM remain.

**Table I**

| Protein                  | Measured mass | Calculated mass |
|--------------------------|---------------|-----------------|
| P1α                      | 10,921 ± 2.2  | 10,924          |
| P1β                      | 10,668 ± 2.2  | 10,668          |
| P2α                      | 10,746 ± 2.0  | 10,746          |
| P2β                      | 11,050 ± 2.6  | 11,050          |
| P9                       | 33,766 ± 4.6  | 33,766          |
| L12-methyl              | 17,777 ± 1.9  | 17,779          |
| L1(-N-terminal Met)      | 24,412 ± 3.0  | 24,414          |
| L1-acetyl               | 24,454 ± 6.4  | 24,453          |
| L27(-N-terminal Met)     | 15,404 ± 1.9  | 15,400          |
| L30β                    | 11,286 ± 0.4  | 11,284          |
| Pentamer                 | 77,291 ± 7.0  | 77,154          |
| Pentamer (minus) P2β     | 66,321 ± 13.0 | 66,104          |

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| Pentamer                 | 77,291 ± 7.0  | 77,154          |
| Pentamer (minus) P2β     | 66,321 ± 13.0 | 66,104          |

* The protein is observed in spectra from the wild type yeast strain.
* The protein is observed in the D5, D7, and D57 strains.
* The calculated mass is taken from Swiss-Prot data base.
* The calculated mass is taken from Ref. 23.
* The standard deviation arises from the fact that at least three charge states were used to measure the mass of the protein.

The observation of proteins L27 and L30 in the mass spectra of ribosomes from mutants strains but not from wild type, where extensive overlap of peaks prevents unambiguous assignment. The presence of a compact or shielded conformation of P0 when bound to the full complement of P1 and P2 proteins and reduction in intensity of P2α is attributed to absorption of these acidic proteins on the filtration device. The observation of proteins L27 and L30 in the mass spectra of ribosomes from D strains implies that when the full complement of the P1/P2 proteins is present in the wild type, L27 and L30 bind more tightly to ribosomal particles. It is also intriguing to note that appreciable dissociation of P0 is observed but would not be predicted based on the fact that it binds more tightly to rRNA than does its prokaryotic counterpart L10 (30). Since P0 is observed here, this implies P0/rRNA interactions are destabilized, presumably because dissociation of the P1 and P2 proteins perturbs the interaction of P0 with rRNA. Similarly, for L1, which in S. cerevisiae forms a protuberance in an equivalent position to the bacterial ribosomal protein L1 and interacts largely with 26 S rRNA (4, 31), we conclude that it has a relatively low surface area of interaction with rRNA, reflecting the ease with which it dissociates from the yeast ribosomal particle.

A number of lines of evidence indicate that the interactions between the four P1/P2 proteins in S. cerevisiae are non-equivalent; there are specific associations between P1β and P2α and P1α and P2β (the proteins absent in strains D7 and D5, respectively) (32, 33). It has been proposed that P1 proteins provide anchorage to the ribosome, whereas the P2 proteins confer functionality to the complex (13), and that P2β is unable to bind the ribosome in the absence of P1α (13). The P1 proteins apparently have a higher affinity for P0 than for P1β and P2p (33). This proposal is supported by the tandem mass spectrometry experiments of the stalk complex reported here. Release of the P2 proteins occurs preferentially to the P1 proteins, and P0 was not observed under these experimental conditions. In strain D7, therefore, the absence of P2β can be explained, since without P1α on the ribosome binding of its "partner," P2β, is destabilized. A different scenario was envisaged for strain D5, where observation of P1α was not thought to depend upon the presence of P2β on the ribosome, but surprisingly P1α was also found to be destabilized. Analysis by IEF of both strains clearly demonstrated, however, that P2β and P1α are present in D7 and D5, respectively. An explanation for this apparent discrepancy is that although present on the ribosome, the protein is significantly destabilized such that the free form is in equilibrium with the intact particle. This equilibrium was found to be
perturbed by absorption of the free proteins on the membrane of the purification device employed to remove storage buffer from ribosomes immediately prior to mass spectrometry, leading to the absorption of free P2β or P1α. Previous investigations have established that the highly acidic nature of the P proteins often prevents efficient recovery; no P proteins were observed in a recent investigation employing mass spectrometry and chromatographic separation (23). We propose, therefore, that although present in the original solution of ribosomes, confirmed by the IEF, the "lone" proteins in the single deletion mutants are significantly destabilized in the absence of their original binding partner.

The possibility that the deletion mutants are heterogeneous, being formed by two types of particles each carrying only two P1/P2 proteins and not necessarily corresponding to the pre-assembled P1/P2 proteins and not necessarily corresponding to the pre-assembled complex but rather to a stable secondary or tertiary structure. The absorption of free P2β has a higher affinity for P0 than does P2α (33), the results presented here indicate that interaction with P2β increases the binding stability of P1α to the ribosome. Our results argue, therefore, that in the absence of P2β, the interactions of P1α with P0 are substantially destabilized leading to loss of P1α. This would suggest that rather than P1 proteins providing anchorage for P2 proteins on the ribosome, the presence of both proteins is required. Recently it has been shown using circular dichroism experiments that P1α and P2β form a stable tertiary structure upon association in solution (14). These results have been interpreted in terms of P1α and P2β being present in the cytoplasm as a stable dimer that can interact directly with the 60 S ribosomal subunit. In the absence of a partner protein, P1α is degraded, whereas P2β accumulates in the cytoplasm (9). Our results are in accord with these findings, because they also point to an increase in affinity of both P1α and P2β for ribosomes when both proteins are present. This implies that assembly of the stalk complex is a cooperative process involving binding with the canonical protein prior to assembly on the ribosome.

CONCLUSIONS

The results from this study have provided clear evidence of the existence of the pentameric stalk and the increased stability of the full complement of the P1/P2 proteins over that of the deletion mutants, as well post-translational modifications of many ribosomal proteins. Taken together, therefore, the ability to monitor dissociation of proteins from such a large and heterogeneous as S. cerevisiae ribosomes has provided new information about interactions and post-translational modifications not observed previously. It is noteworthy, from a methodological point of view, that this so called "top-down" proteomics approach (34), involving introduction of the intact ribosome, has yielded information about populations of proteins with more than one modification; these are difficult to define using standard protocols where the intact protein is cleaved. The ability to monitor interactions in complexes such as the pentameric stalk while still attached to the ribosome is a powerful adjunct to this top-down approach. Interestingly, the stalk complex from prokaryotic ribosomes has been the most difficult to define using established structural biology methods, because of its inherent conformational heterogeneity. This mass spectrometry approach is therefore likely to be of widespread utility in defining macromolecular complexes of this magnitude, particularly for dynamic complexes where detailed structural information is difficult to obtain.

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