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# Structure and Activities of a Variant Ubiquitin Sequence from Bakers' Yeast†

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ABSTRACT: Ubiquitin is an extremely conserved protein, with an identical sequence throughout the animal kingdom. However, the gene sequence of the yeast protein [Ozkaynak, E., Finley, D., & Varshavsky, A. (1984) *Nature (London) 312*, 663–666] predicts three amino acid differences. This implies that some functions or binding interactions of ubiquitin are different in yeast and animal cells. In an effort to define these differences, ubiquitin has been purified to homogeneity from bakers' yeast and characterized. Amino acid analysis of the protein and the isolated tryptic peptides confirms the primary structure of this protein as predicted from the gene sequence. This result indicates that the gene sequenced is the transcriptionally active gene from yeast. The conformation of yeast ubiquitin is similar to human ubiquitin as judged by circular dichroism, sensitivity to trypsin, and Stokes radius. Yeast and animal ubiquitins show identical activities in supporting ubiquitin-dependent protein degradation and in the ATP–pyrophosphate exchange reaction catalyzed by the purified ubiquitin-adenylating enzyme. Thus, the three conservative amino acid differences between yeast and animal ubiquitins have very little effect on the structure of ubiquitin or its activity in the ubiquitin-dependent proteolytic system. These results suggest that at least some of the evolutionary pressure preventing sequence variation among animal ubiquitins stems from one or more of its nonproteolytic functions.

Ubiquitin is the most highly conserved protein known, with an identical sequence from animals as diverse as insects and humans (Gavalanes et al., 1982; Schlesinger & Goldstein, 1975). Even the protein from oat is identical in 73 of 76 residues (Vierstra et al., 1985, 1986). Recently, the gene sequence of yeast ubiquitin has been reported (Ozkaynak et al., 1984). The protein coded for by this gene has three conservative amino acid substitutions in the amino-terminal third of the molecule. The yeast gene sequence suggests that ubiquitin is synthesized as a polyprotein containing multiple copies of the mature protein, no intervening sequences, and a single asparagine as a carboxyl-terminal extension. The ubiquitin cDNA from humans also shows that ubiquitin is synthesized with a carboxyl-terminal extension (Lund et al., 1985), 80 amino acids in this case. Additionally, there are multiple, larger mRNA species present, which may indicate that the human protein is also synthesized as a polyprotein. Finally, there are multiple mRNA species of different sizes in *Xenopus* (Dworkin-Rastl et al., 1984). This has been shown to be due to the same arrangement of tandem repeats of the ubiquitin coding sequence, but there is no evidence for a C-terminal extension in this organism. Thus, even though the gene structure of these organisms is quite similar, the selective pressure that maintains absolute conservation of sequence in the animal proteins must be different from that in yeast.

One possible explanation for this difference is that the function(s) of ubiquitin may vary between yeasts and animals. Ubiquitin has been suggested to act as an immunostimulating peptide (Goldstein et al., 1975), a regulator of chromatin structure (Matsui et al., 1979) and the cell cycle (Finley et al., 1984), in the heat-shock response (Finley et al., 1984; Levinger & Varshavsky, 1982), in the structure and regulation of cell surface receptors (Siegelman et al., 1986), and as a cofactor in ATP-dependent protein degradation (Wilkinson et al., 1980; Wilkinson & Audhya, 1981). If one or more of the functions of ubiquitin are different between these organ-

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isms, some of the selective pressure maintaining the animal sequence may be absent in yeast.

Our interests have centered on the role of ubiquitin in ATP-dependent protein degradation (Ciechanover et al., 1984). In this system, ubiquitin is conjugated to a variety of cellular proteins via a peptide bond between the carboxyl terminus of ubiquitin and the $\alpha$- or $\epsilon$-amino groups of the target proteins (Hershko et al., 1984). This conjugation is thought to mark the proteins for degradation by proteases of the system. Alternatively, the nascent isopeptide bond can be hydrolyzed by an "isoamidase" to yield the constituent proteins (Hershko et al., 1980). The partition of conjugates between these two pathways may be a proofreading function, designed to assure that only abnormal proteins are degraded by this system. Recent evidence has suggested that the conformation of ubiquitin may play a critical role in the partition of these conjugates (Cox et al., 1986a). Thus, the protein–protein interactions between ubiquitin and the conjugated protein may determine the flux through the two different pathways.

One of the amino acid differences observed between the yeast and the animal ubiquitins is a proline-to-serine substitution at residue 19 (Ozkaynak et al., 1984). This residue is at the end of two strands of anti-parallel $\beta$ sheet forming one face of the molecule (Vijay-Kumar et al., 1985). It might be expected that the replacement of proline-19 by serine would affect the conformation or mobility of this portion of the ubiquitin molecule.

To examine the structure and function of this naturally occurring variant sequence, we have isolated and characterized the protein from yeast. The results demonstrate that the sequenced gene is transcriptionally active in yeast and the resultant protein is very similar to the animal protein.

## Materials and Methods

*Materials.* Dried active bakers' yeast (Fleischmann's, Nabisco Brands Inc., East Hanover, NJ 07936) was obtained from a local bakery. Bovine ubiquitin was isolated from erythrocytes as described previously (Haas & Wilkinson, 1985). Fraction II was purified (Ciechanover et al., 1978) from rabbit reticulocytes (Pel-Freez Biologicals, Rogers, AR 72756), and ubiquitin activating enzyme was purified from fraction II (Haas et al., 1982). Trypsin (TPCK treated from Sigma Chemical Co., St. Louis, MO 63178) was further purified by adsorption and elution from immobilized soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO 63178) according to the manufacturer's instructions. Columns for HPLC were purchased from IBM Instruments, Wallingford, CT 06492 (5-$\mu$m Octyl, 0.45 × 15 cm) and Bio-Rad Laboratories, Richmond, CA 94804 (Biosil ODS-5S, 0.4 × 25 cm). All other chemicals were reagent-grade or better from commercial sources.

*Purification of Yeast Ubiquitin.* Dried yeast (30 g) was hydrated by slow addition to 250 mL of rapidly stirred buffer (10 mM Tris, pH 7.5, 1 mM DTT). After being stirred for 10 min at room temperature, the yeast were broken by homogenization with glass beads (Bead-Beater, Biospec Products, Bartlesville, OK 74005). Five cycles of 1 min were employed with the chamber cooled in ice–water. The homogenate was decanted, and the beads were washed with an additional 200 mL of water and removed by straining through cheesecloth. Typically, 10 batches were processed as above, and the homogenates and washes were pooled. The pooled material was then centrifuged at 10000$g$ for 30 min and the precipitate discarded. The supernatant was transferred to a 4-L glass beaker and stirred while being heated on a hot plate (Corning, PC-351). When the temperature reached 90 °C (about 45 min), the beaker was removed from the heat source and cooled to room temperature. Denatured protein was removed by centrifugation at 10000$g$ for 30 min. The supernatant was brought to 5% (w/v) trichloroacetic acid by addition of a 100% (w/v) solution with rapid stirring. After the protein was allowed to precipitate overnight at 4 °C, the bulk of the supernatant was removed by means of a siphon. The remaining slurry of precipitated protein was centrifuged for 30 min at 15000$g$ and the supernatant discarded. The pellet was suspended in a minimal volume of water and the pH adjusted to 7.6 with ammonium hydroxide. After dialysis against water, the pH of the solution was adjusted to 9.0 with ammonium carbonate, and any insoluble protein was removed by centrifugation. This sample was applied to a 2 × 50 cm column of DEAE-52 equilibrated with 20 mM ammonium bicarbonate, pH 9.0, and eluted with the same buffer. Yeast ubiquitin eluted between 2 and 4 column volumes (the major absorbance peak elutes between 1 and 2 column volumes). Ubiquitin was located by HPLC as described below. The pooled eluate from the DEAE-52 was adjusted to pH 4.5 with acetic acid and applied to a 2 × 20 cm column of CM-52 equilibrated with 50 mM ammonium acetate and washed with 2 column volumes of the same buffer. Ubiquitin was eluted by 50 mM ammonium acetate, pH 5.5 (as a broad peak shortly after the pH reached 5.5), and located by HPLC. The ubiquitin-containing fractions were pooled, diluted 3-fold with water, and adjusted to pH 9.4 with ethanolamine. This fraction was applied to a 0.9 × 25 cm column of QAE-Sephadex A-25 equilibrated with 75 mM ethanolamine–acetate, pH 9.4, and washed with 2 column volumes of the same buffer. Ubiquitin was eluted with 50 mM sodium acetate in the equilibration buffer. The fractions containing ubiquitin were pooled and dialyzed extensively vs. water, and the protein concentration was determined (Lowry et al., 1951). The purified protein was stored frozen at −20 °C.

*Assays.* Yeast ubiquitin was quantitated by an HPLC method as follows. An initial sample of partially purified yeast ubiquitin was prepared by breaking the yeast cells as described above and submitting the soluble proteins to the purification procedure described for human ubiquitin (Haas & Wilkinson, 1985). Analysis of the purified material demonstrated that it was active in stimulating ATP-dependent proteolysis. Chromatography of this fraction on a C-8 column equilibrated with 25 mM sodium perchlorate–12.5 mM perchloric acid in 42% acetonitrile revealed one major protein peak eluting at 6 min (flow rate of 2 mL/min, absorbance at 205 nm monitored). Chromatography of this sample on the QAE-Sephadex A-25 column described above resulted in a homogeneous protein that was subjected to protein determination and used as a standard in subsequent studies. During optimization of the purification procedure, yeast ubiquitin was quantitated by chromatography of an aliquot on the C-8 column under the conditions described above, and comparison of the peak heights to those obtained with the standard sample was made. Crude samples could be treated with 7% (v/v) perchloric acid and centrifugation without loss of ubiquitin from the supernatant. Injection of the perchloric acid supernatant resulted in much less interference from contaminating proteins in this HPLC determination.

The rate of trypsin-catalyzed cleavage of the carboxyl-terminal diglycine was monitored by HPLC. The reaction mixture contained 0.2 mg/mL yeast or bovine ubiquitin, 0.01 mg/mL affinity purified trypsin, and 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0. At the indicated times of incubation at 37 °C, aliquots were

Table I: Purification of Yeast Ubiquitin[a]

| step | vol (mL) | protein (mg) | ubiquitin (mg) | x-fold purification | purity (%) | % yield |
|---|---|---|---|---|---|---|
| homogenate[b] | 3200 | 26000 | 46.0[c] | 1 | 0.18 | 100 |
| heat step | 2800 | 9080 | 11.6[c] | 1 | 0.14 | 25 |
| TCA pellet | 75 | 3510 | nd[d] | nd | nd | nd |
| DE-52 eluate | 125 | 82 | 8.5 | 58 | 10.4 | 19 |
| CM-52 eluate | 100 | 15 | 8.2 | 304 | 54.7 | 18 |
| QAE-A25 eluate | 40 | 7.8 | 7.8 | 555 | 100 | 17 |

[a] Protein was determined by the method of Lowry et al. (1951), and ubiquitin was determined by HPLC as described under Materials and Methods. Values shown are averages from three separate preparations. [b] From 300 g of dry active bakers' yeast. [c] Assayed in the supernatant after addition of 7% (v/v) perchloric acid and centrifugation of the precipitated protein. [d] Not determined.

removed and chromatographed on the C-8 column as described above. Cleavage of the diglycine results in increased retention under these conditions, and the amounts of native and cleaved protein were determined by integration of the areas of the respective peaks.

Stimulation of ATP-dependent protein degradation was measured as described previously (Evans & Wilkinson, 1985) with $^{125}$I-reduced carboxymethylated bovine serum albumin. ATP–$PP_i$ exchange catalyzed by the purified ubiquitin activating enzyme was measured in the presence of 1 mM AMP (Haas et al., 1982).

*Other Methods.* Bovine and yeast ubiquitin were digested with trypsin in the presence of urea, the peptides were separated by HPLC, and the purified peptides were subjected to amino acid analysis as described previoulsy (Cox et al., 1986b). Alternatively, the tryptic peptides were carbamylated at 50 °C in the presence of 50 mg/mL KCNO (Stark, 1967) and then chromatographed on HPLC.

## Results

*Purification of Yeast Ubiquitin.* The reported gene sequence for yeast ubiquitin (Ozkaynak et al., 1984) indicated this protein should have the same molecular weight, isoelectric point, and general properties as the protein from animal tissues. Therefore, we first subjected homogenates of bakers' yeast to the purification scheme used for the human protein (Haas & Wilkinson, 1985). This procedure yielded a preparation of protein that was active in stimulating protein degradation in the reticulocyte system (Evans & Wilkinson, 1985). Analysis of this protein by HPLC showed predominantly one peak that eluted slightly later than human ubiquitin (see Materials and Methods). This preparation of protein was used as an HPLC standard for the subsequent optimization of the purification procedure.

Table I presents the quantitative analysis of the final purification procedure. Ubiquitin was purified over 500-fold in an overall yield of 17%. After initally breaking the cells by homogenization with glass beads, the procedure is similar to that for the human protein. The largest losses occur with the heat treatment step, but this step appears to be necessary to remove contaminating proteins that subsequently copurify with ubiquitin. An additional step, chromatography on QAE-Sephadex, is also required to obtain homogeneous protein. The yield of ubiquitin is about 27 mg/kg of dried active bakers' yeast.

*Amino Acid Analysis and Peptide Mapping.* When the purified protein is subjected to acid hydrolysis and amino acid analysis, the data shown in Table II are obtained. There is excellent agreement between the observed amino acid composition and that expected on the basis of the gene sequence. The residues most indicative of the predicted differences are serine (5 expected, 4.8 found, 3 for animal ubiquitin), proline (2 expected, 2.1 found, 3 for animal ubiquitin), and alanine (1 expected, 1.2 found, 2 for animal ubiquitin).

Table II: Amino Acid Analysis of Yeast Ubiquitin

| amino acid | residues expected[a] | residues found[b] |
|---|---|---|
| Asx | 8 | 7.8 |
| Thr | 7 | 6.7 |
| Ser | 5 | 4.8 |
| Glx | 11 | 11.0 |
| Pro | 2 | 2.1 |
| Gly | 6 | 6.4 |
| Ala | 1 | 1.2 |
| Val | 4 | 4.1 |
| Met | 1 | 1.1 |
| Ile | 7 | 7.1 |
| Leu | 9 | 9.6 |
| Tyr | 1 | 1.0 |
| Phe | 2 | 1.9 |
| His | 1 | 1.1 |
| Lys | 7 | 7.5 |
| Arg | 4 | 3.9 |

[a] From the gene sequence (Ozkaynak et al., 1984). [b] Average of duplicate 24- and 48-h hydrolysates with bovine ubiquitin hydrolysates as the integration standard.

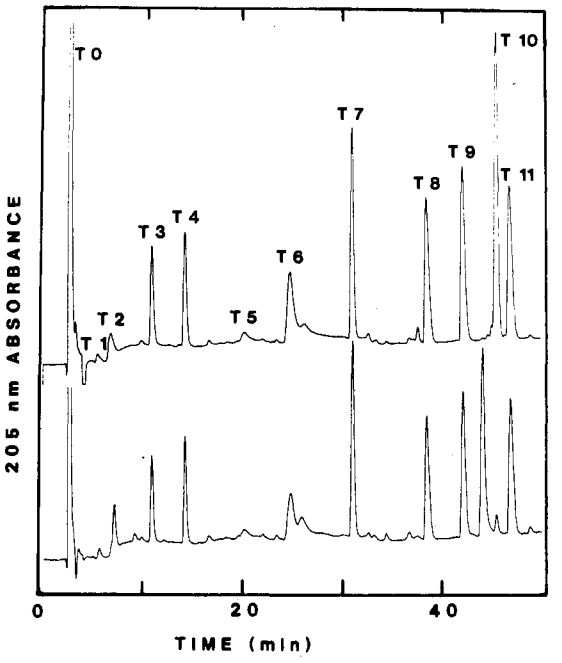


FIGURE 1: Tryptic peptide maps of bovine and yeast ubiquitin. Digestions and HPLC were performed as described earlier (Cox et al., 1986). The upper panel shows the chromatogram obtained from digestion of the bovine protein, and the lower panel shows the chromatogram obtained from digestions of the yeast protein. The peptides are designated T0–T11.

In order to confirm the expected amino acid substitutions, we have subjected the purified protein to tryptic hydrolysis and peptide mapping on HPLC (Cox et al., 1986b). Figure 1 shows the resultant chromatograms for bovine and yeast ubiquitin. It can be seen that with the exception of peptide

Table III: Amino Acid Analysis of Peptide T10 from Bovine and Yeast Ubiquitin[a]

| amino acid | bovine | | yeast | |
|---|---|---|---|---|
| | expected | found | expected | found |
| Asx | 2 | 2.0 | 3 | 3.2 |
| Thr | 3 | 2.7 | 3 | 2.6 |
| Ser | 1 | 1.1 | 2 | 2.0 |
| Glx | 3 | 2.9 | 2 | 1.8 |
| Pro | 1 | *b* | 0 | *b* |
| Val | 2 | 1.5 | 2 | 1.5 |
| Ile | 2 | 1.3 | 2 | 1.1 |
| Leu | 1 | 0.9 | 1 | 1.1 |
| Lys | 1 | 0.6 | 1 | 0.5 |

[a] The proteins were digested with trypsin, and the peptides were purified by HPLC and hydrolyzed for 24 h before amino acid analysis. Values for valine, isoleucine, and lysine are low due to incomplete hydrolysis. [b] Not determined.

T10 the pattern is identical with these two proteins. Under the conditions used for digestion, two tryptic cleavage sites are resistant in the bovine protein. Peptide T2 contains Arg-74 in a resistant linkage, and peptide T6 contains Lys-36 in a resistant linkage. Longer times of digestion lead to hydrolysis of this latter bond and appearance of peptides T5 and T1. The similar extent of this slower cleavage with both proteins is also evidence for the similarity of sequence of these peptides between the two species. The peptides from the yeast protein were purified by HPLC and subjected to acid hydrolysis and amino acid analysis. With the exception of peptide T10, the amino acid compositions were identical with those of the corresponding peptides from the bovine protein. Peptide T10 contains residues 12–27 of ubitquitin and is predicted to have two amino acid changes in the yeast protein. The amino acid composition of peptide T10 from yeast ubiquitin is compared with that from bovine ubiquitin in Table III. It can be seen that the amino acid composition confirms the two expected amino acid substitutions in this peptide. The only peptide not resolved and accounted for by this analysis is the dipeptide Ala-Lys (residues 28 and 29), which elutes in the void volume (T0) under these conditions. Carbamylation of the peptides (Stark, 1967) before chromatography results in an increased retention for peptide T0, and in control experiments we have been able to quantitatively detect the dipeptide Ala-Lys in digests of the bovine protein. The corresponding peptide in the yeast protein is predicted to be Ser-Lys. Therefore, digests of the yeast protein were subjected to carbamylation and chromatography as above. No Ala-Lys could be detected in these digests, indicating that the corresponding yeast peptide is different. This result, combined with the total amino acid analysis of the purified protein, confirms the predicted change from alanine to serine at residue 28 of the yeast protein.

*Physical Characterization.* The purification scheme demonstrates that yeast ubiquitin has the same ionic characteristics as animal ubiquitin, since it behaves identically in the three ion-exchange steps. Additionally, we have found that the protein migrates in the same position as animal ubiquitin when chromatographed on Sephadex G-50 (data not shown). To examine the similarity of conformations of these two proteins, circular dichroism spectra were obtained. The spectra of these two proteins were found to be identical, indicating a similar content of helical structure. To examine the conformation mobility of the proteins, both were subjected to digestion by trypsin (5% w/w) at pH 8.0. Under these conditions, the native bovine protein is converted to a 74 amino acid fragment and the carboxyl-terminal diglycine (Wilkinson & Audhya, 1981). No further cleavages occur, in spite of the seven lysines and three arginines present in the large fragment. When yeast

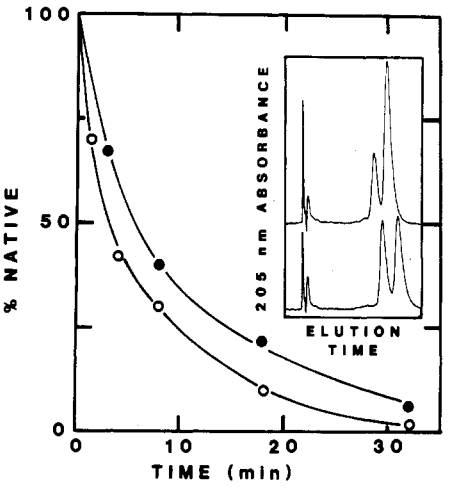


FIGURE 2: Tryptic cleavage of carboxyl-terminal diglycine from bovine and yeast ubiquitin. The proteins were digested with trypsin at 37 °C for the indicated times. Aliquots were chromatographed on the C-8 column (Materials and Methods), and the amount of native and cleaved protein was determined by integration of the areas under the peaks. Closed circles show the results obtained with the bovine protein and open circles those for the yeast protein. The inset shows chromatograms obtained during the digestion: bovine ubiquitin after 19 min of digestion (upper trace) and yeast ubiquitin after 5 min of digestion (lower trace). In both chromatograms, the earlier eluting peak is the native protein, and the later eluting peak is the cleavage product.

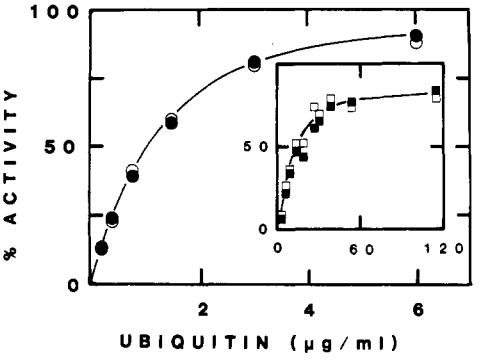


FIGURE 3: Activity of bovine and yeast ubiquitin in the reactions of protein degradation. Both proteins were assayed for their activity in supporting ATP-$PP_i$ exchange (circles) catalyzed by the purified ubiquitin-activating enzyme and activity in stimulating ATP-dependent proteolysis (squares) catalyzed by reticulocyte fraction II. Open symbols are data from the yeast protein and closed symbols those from the bovine protein. Results are expressed as a percentage of activity observed with saturating amounts of the bovine protein.

and bovine ubiquitin are subjected to trypsin and aliquots of the digestion mixtures are analyzed by HPLC (Figure 2), it can be seen that similar behavior is observed for both of these proteins. Over the first 30 min, both proteins are converted to later eluting peaks with similar area to that of the starting compound. Subsequent incubation with trypsin causes no further change in this profile. Thus, both proteins have similar conformations and stability.

*Activity of Yeast Ubiquitin in the Protein Degradation System.* Figure 3 compares the activity of the yeast and bovine proteins in stimulating ATP–pyrophosphate exchange catalyzed by the purified ubiquitin-activating enzyme from rabbit reticulocytes (Haas et al., 1982). These two proteins show virtually identical activity in this assay. This reaction is the first step in the conjugation of ubiquitin to proteins. Thus, the amino acid substitutions found in yeast have no effect on the rate of this activation step. Two further reactions are necessary to form conjugates (Hershko et al., 1983), the

transesterification by a thiol group of the ubiquitin carrier protein (E2) and the amination of this thiol ester with protein amino groups catalyzed by the conjugating enzyme (E3). These enzymes have not yet been obtained in homogeneous form, so assays of these activities were not attempted.

To examine the activity of yeast ubiquitin in overall protein degradation (i.e., activation, conjugation, and proteolysis), we measured the ability of yeast ubiquitin to stimulate proteolysis catalyzed by the reticulocyte system. Figure 3 (inset) compares the activity of yeast and bovine ubiquitin in stimulating ubiquitin-dependent protein degradation catalyzed by the reticulocyte system. There is no difference in the ability of these two proteins to stimulate protein degradation. Thus, yeast ubiquitin is recognized by the reticulocyte enzymes and shows kinetic behavior identical with that observed with animal ubiquitins.

## Discussion

This work descirbes the purification and characterization of a naturally occuring variant sequence of ubiquitin. This protein has an identical sequence throughout the animal kingdom (Vijay-Kumar et al., 1985). The usual interpretation of such conservation of sequence is that all portions of the molecule are required for important physiological functions. Thus, it is possible that one or more of the functions of ubiquitin are different in yeasts and animals. Purification of this protein was initiated to confirm the predicted amino acid sequence (Ozkaynak et al., 1984) and to begin comparative studies which may define these different roles in yeasts and animals. A similar study has been initiated comparing the animal and plant ubiquitins (Vierstra et al., 1985, 1986).

The purification procedure for yeast ubiquitin (Table I) is very similar to that used for animal erythrocyte ubiquitin. One additional step, anion-exchange chromatography on QAE-Sephadex A-25, is required to obtain homogeneous protein. The yield of ubiquitin (27 mg/kg of dried yeast) and the ease of obtaining large amounts of dried yeast make this an attractive procedure for purification of significant amounts of protein. It should be noted that the amount of ubiquitin present in the homogenates is similar to that found in most other eucaryotic cells (Goldstein et al., 1975) and amounts to about 0.2% of the soluble protein in yeast.

The purified protein is identical with that predicted by the gene sequence given previously (Ozkaynak et al., 1984). Purification of the tryptic peptides by HPLC, comparison of these peptides with those from bovine ubiquitin (Figure 1), and amino acid analysis of the protein and purified peptides (Tables II and III) confirm the predicted sequence. Additionally, ubiquitin from yeast and animals shows identical behavior in ion-exchange separations at pH 5.5 and 9.4, indicating that they have very similar isoelectric points and charge densities. In separate experiments we have observed that a single charge difference is easily observed in these ion-exchange steps. Thus, the amide residues are accounted for as well.

Yeast ubiquitin is conformationally similar to bovine ubiquitin. The two proteins comigrate on Sephadex G-50 chromatography, indicating that they have a similar Stokes radius, and show identical circular dichroism spectra, indicating a similar helical content (data not shown). The two proteins show identical rates of tryptic cleavage at the arginine-74 bond in aqueous solution and marked stability to the cleavage at other lysine and arginine bonds (Figure 2). This stability to trypsin is a function of the conformational stability of the protein, since all of these bonds are cleaved in the presence of urea (Figure 1). Finally, we have been able to crystallize ubiquitin from yeast, and preliminary results[1] suggest it has a folding pattern identical with that of the animal protein (Vijay-Kumar et al., 1985). One can distinguish between these two proteins however by HPLC (Figure 2). Both the 74 and 76 amino acid sequences of yeast ubiquitin elute slightly later than the corresponding bovine proteins (inset to Figure 2).

The above results demonstrate that the amino acid substitutions present in the yeast sequence do not perturb the structure or stability of ubiquitin. Two of the substitutions, Ala to Ser at residue 28 and Glu to Asp at residue 24, would not be predicted to have large effects on the structure. The third substitutions Pro to Ser at residue 19, does not seem to have a significant effect either. This residue is involved in a turn between the second $\beta$ sheet and the subsequent helical segment (Vijay-Kumar et al., 1985). In spite of their differences, both Pro and Ser are easily accommodated in turn structures.

The functional characteristics of yeast and animal ubiquitin in protein degradation are also very similar. These proteins show identical kinetic behavior in the adenylation of ubiquitin catalyzed by the purified reticulocyte ubiquitin-activating enzyme (Figure 3) and in overall protein degradation catalyzed by the reticulocyte system (Figure 3, inset). Thus, the enzymes of the reticulocyte system recognize and use the yeast and animal proteins equally. The converse also appears to be true. The enzymes from yeast have been shown to recognize and conjugate human ubiquitin to endogenous yeast proteins (Ozkaynak et al., 1984). These results imply that the enzymes of this proteolysis system are similar in yeast and animals.

It is interesting to note that the three amino acid changes are clustered in the primary and tertiary structure of ubiquitin. Residue 19 is at the carboxyl-terminal end of the single helix of ubiquitin, while residues 24 and 27 are both on the exposed face of this ampithetic helix. Thus, the differencces between yeast and animal ubiquitin are localized to a small portion of the surface of the molecule. Recently, the structure of oat ubiquitin has been reported (Vierstra et al., 1986). This molecule also has three amino acid differences from the animal protein: Pro vs. Ser at residue 19, Glu vs. Asp at residue 28, and Ala vs. Ser at residue 52. This latter residue is also in the same localized region of the folded molecule. Thus, the differences in the three sequences known are restricted to a small region of the ubiquitin molecule. All three of these proteins show similar activity in supporting ubiquitin-dependent proteolysis. It would appear that the enzymes of the ATP-dependent proteolysis system do not distinguish small differences in this portion of the ubiquitin molecule.

Since this region of the molecule (and indeed the entire molecule) is so highly conserved in the animal ubiquitins, it is unclear why these substitutions arose during evolution from the more primitive ubiquitin structure represented by the yeast and plant proteins. Clearly, the selective pressure on this portion of the molecule is different in these organisms. On the basis of the results presented above, that selective pressure would seem to involve one of ubiquitin's nonproteolytic functions. It may be that ubiquitin has a function in animals that is not utilized in lower eucaryotes. This may be related to the immunostimulating properties of ubiquitin (Goldstein et al., 1975) and the recent observation that ubiquitin is present on the surface of animal cells (Siegelman et al., 1986). The evolution of such a higher function would exert and maintain selective pressure on this region of the molecule that does not exist in the lower eucaryotes. Alternatively, it is possible that

[1] W. J. Cook and K. D. Wilkinson, unpublished results.

some of the enzymes of a common function have evolved enough to require a slightly different structure in this region of the ubiquitin molecule.

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# A Unique Pair of Zinc Binding Sites in the Human $\alpha_2$-Macroglobulin Tetramer. A $^{35}Cl$ and $^{37}Cl$ NMR Study†

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ABSTRACT: $^{35}Cl$ NMR has been used to demonstrate that human $\alpha_2$-macroglobulin tetramer possesses a unique pair of zinc binding sites. Zinc bound at these sites does not affect the $^{35}Cl$ NMR line width of free $Cl^-$. Additional lower affinity zinc sites exist that bind chloride weakly and cause broadening of the free chloride resonance through fast exchange with bound chloride. Using both $^{35}Cl$ and $^{37}Cl$ relaxation measurements it has been shown that chloride bound at these sites has an internal correlation time of 5.1 ns and a quadrupolar interaction, $\chi$, of 4.2 MHz with zinc. Manganese binds to apo-$\alpha_2$-macroglobulin analogously to zinc. $\alpha_2$-Macroglobulin that has been reacted with methylamine still possesses two classes of zinc sites per tetramer, but their relative affinities differ more than for unreacted $\alpha_2$-macroglobulin. These data are discussed with respect to possible models for the subunit arrangement in the tetramer.

$\alpha_2$-Macroglobulin is present in human serum at a concentration of about 2.5 g $L^{-1}$ and thus represents about 45% by weight of the major class of serum proteins that are characterized as proteinase inhibitors (Harpel & Brower, 1983). It also possesses bound zinc that has been quantitated as being in the range 3.9-9.2 mol of zinc/mol of $\alpha_2$-macroglobulin (Parisi & Vallee, 1970). As a consequence it represents the second largest locus, after serum albumin, of zinc in the blood, accounting for 30-40% of the total (Parisi & Vallee, 1970; Vessel & Bearn, 1957). The zinc appears to be tightly bound, since exposure to $^{65}Zn$ does not lead to exchange with endogenous zinc (Parisi & Vallee, 1970) and therefore $\alpha_2$-

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