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The Complete Amino Acid Sequence of Ubiquitin, an Adenylate Cyclase Stimulating Polypeptide Probably Universal in Living Cells†

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ABSTRACT: The complete amino acid sequence was determined for bovine ubiquitin, an adenylate cyclase stimulating polypeptide, which is probably represented universally in living cells. Ubiquitin has a molecular weight of 8451 and consists of a single polypeptide chain containing 74 amino acid residues. It contains four arginine residues but no cysteine or trytophan residues. The first 61 amino acid residues were obtained by automated Edman degradations. Tryptic digestion of maleated ubiquitin yielded four peptide fragments that were resolved by molecular sieve chromatography and coded in order of decreasing chain length (MT-1, MT-2, MT-3, and MT-4). The automated sequenator determinations on native ubiquitin provided overlapping sequence data for three of these fragments that gave an order of MT-1, MT-3, and then MT-2. Peptide MT-4, a dipeptide, was therefore assigned to the C terminus, and the placement of peptide MT-2 was corroborated by analysis of data from carboxypeptidase digestions of maleated ubiquitin. Peptide MT-2 was demaleated and sequenced by manual Edman degradations through a single lysine residue. it was cleaved at this residue with trypsin, and the two resultant peptides were separated by ion-exchange chromatography. Manual sequencing of the C-terminal demaleated tryptic peptide of MT-2 completed the sequence of MT-2 and that of native ubiquitin. The sequence of ubiquitin was further confirmed and supported by amino acid and partial sequence analysis of fragments obtained by digestion of maleated ubiquitin with chymotrypsin or staphylococcal protease.

Ubiquitin (formerly ubiquitous immunopoietic polypeptide or UBIP) is an 8451-dalton polypeptide first isolated from bovine thymus but subsequently found to be present in the cells of all tissues studied and, indeed, in the cells of most living organisms, as judged from examples of animals, yeast, bacteria, and higher plants (Goldstein et al., 1975). Ubiquitin was purified in the course of isolation of thymopoietin, a polypeptide hormone of the thymus (Goldstein, 1974). Thymopoietin specifically induces the differentiation of thymocyte precursors, presumably by combining with a receptor on the prothymocyte membrane and triggering adenylate cyclase activation (Basch and Goldstein, 1974; Scheid et al., 1975). Ubiquitin can mimic thymopoietin-induced differentiation, but its action is not restricted to prothymocytes, since it can activate B-cell differentiation (Goldstein et al., 1975; Scheid et al., 1975) and adenylate cyclase in a wide variety of tissues (Bitensky and Goldstein, 1975). The stimulation of adenylate cyclase by ubiquitin is inhibited by propranolol (Goldstein et al., 1975; Scheid et al., 1975), and it is therefore presumed to have a β-adrenomimetic active site and to act via a β-adrenergic receptor. Ubiquitin shows a high degree of evolutionary conservation, exhibiting close functional and immunological similarity when isolated from such diverse origins as cells of mammals and higher plants (Goldstein et al., 1975). In a preliminary study comparing the first eight amino acid residues of the N-terminal sequence of bovine and celery ubiquitin, there were six identical residues and one substitution at position 1; position 4 of celery ubiquitin was not identified.

The exact function of this adenylate cyclase stimulating polypeptide remains unknown; that it subserves some function vital in the living organism may be inferred from its extraordinary evolutionary conservation. The likely structural

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similarity of the ubiquitin binding site to the low molecular weight ligand epinephrine raises further points of interest. This report presents the complete amino acid sequence of bovine ubiquitin.

Experimental Section

Materials

Ubiquitin was isolated from bovine thymus as described by Goldstein et al. (1975). Trypsin, α-chymotrypsin, carboxypeptidase A, and carboxypeptidase B were purchased from Worthington Biochemicals. Staphylococcal protease was a gift from Dr. G. Drapeau, Department of Microbiology, University of Montreal, Canada. Sequenator reagents, of "Sequenal" grade, were purchased from Beckman Instruments. Fluorescamine (Hoffman-La Roche) was initially a gift of Dr. S. Udenfriend.

Methods

Amino Acid Analysis. Polypeptides were hydrolyzed in 6 *N* HCl containing 0.05% (v/v) β-mercaptoethanol at 110° in vacuo for 24 hr. Amino acids were identified on a Model 121 Beckman amino acid analyzer by the method of Spackman et al. (1958).

Automated Sequenator Analysis. This was performed by the method of Edman and Begg (1967) in which a double-cleavage Quadrol program was used. One micromol of ubiquitin was dissolved in 0.05 *M* NH_4HCO_3 (pH 9.6) and lyophilized in the sequenator cup. Coupling with phenyl isothiocyanate was performed twice before initiating automated sequencing.

Manual Edman Degradations. Manual phenyl isothiocyanate degradations were performed by the three-stage method of Edman (1960) as modified by Sauer and Niall (1974). Coupling of peptide was carried out under nitrogen for 30 min at 54° in 100 μl of 0.4 *M* dimethylallylamine in propanol–water (60:40, v/v) previously adjusted to pH 9.5 with trifluoroacetic acid. After coupling, a single extraction with 0.2 ml of benzene was performed, and the organic phase discarded. Cleavage was accomplished with 75 μl of trifluoroacetic acid under nitrogen at 54° for 3–5 min. This was followed by extraction of thiazolinone products with 0.25 ml of benzene.

The thiazolinones were converted to their more stable Pth[1] amino acid isomers in 0.2 ml of 1 *M* HCl at 80° for 10 min.

Identification of Pth Amino Acids. This was carried out by gas chromatography (Pisano and Bronzert, 1969) and by thin-layer chromatography (Edman, 1970). Pth-His and Pth-Arg were identified by thin-layer chromatography on silica gel using the solvent system chloroform–methanol–heptafluorobutyric acid (70:30:0.5) (unpublished data). After development of the thin-layer plate, intensity of the Pth amino acids was enhanced by vapors of iodine (Ingles et al., 1974). The plate was transilluminated by ultraviolet (uv) light at 254 nm and photographed for permanent records.

Maleation of Native Ubiquitin. Maleic anhydride in 1,4-dioxane was added at a 30-fold excess over the free amino groups of the polypeptide, which was dissolved in 0.2 *M* sodium borate (pH 9.25). Maleic anhydride was added stepwise over a 3-hr period, pH 9.2 being maintained with 5 *N* NaOH. Maleated ubiquitin was then desalted by molecular sieve chromatography in 0.2 *M* NH_4HCO_3 (pH 8.2) on P-2 Bio-Gel (Bio-Rad Laboratories).

Demaleation of Selected Peptides. This was achieved by dissolving the peptide in 1 *N* formic acid, heating at 80° for 50 min, diluting with water, and lyophilizing.

Enzymatic Digestion and Purification of Peptides. TRYPTIC PEPTIDES. Maleated ubiquitin was dissolved in 0.2 *M* NH_4HCO_3 (pH 8.2) and digested with trypsin at an enzyme–substrate ratio of 1:100 (w/w) for 3 hr at 37°. The digest was then acidified to pH 3 with 6 *N* acetic acid and lyophilized. The tryptic peptides were separated by molecular sieve chromatography on P-6 Bio-Gel equilibrated with 0.2 *M* NH_4HCO_3 (pH 8.2) in a column 140 × 1.2 cm.

Peptide fragment MT-2 from this digestion was demaleated (dMT-2) and further digested with trypsin as described. The two resultant peptides (T1-dMT-2 and T2-dMT-2) were separated by ion-exchange chromatography on DEAE-Sephadex A-25 equilibrated with 0.05 *M* NH_4HCO_3 (pH 8.0) in a column 5 × 0.5 cm utilizing a gradient to 0.5 *M* NH_4HCO_3 with a constant pH of 8.2.

CHYMOTRYPTIC PEPTIDES. Maleated ubiquitin was dissolved in 0.2 *M* NH_4HCO_3 (pH 8.2) and incubated with chymotrypsin for 3 hr at 37° at an enzyme–substrate ratio of 1:100. After acidification and lypohilization of the chymotryptic digest, peptide fragments were partially purified on P-6 Bio-Gel. Selected peptides were then fully purified on DEAE-Sephadex A-25 as described.

STAPHYLOCOCCAL PROTEASE PEPTIDES. This enzyme cleaves the peptide bond of the α-carboxyl group of glutamyl residues (Houmard and Drapeau, 1972). Maleated ubiquitin was incubated with staphylococcal protease in 0.2 *M* NH_4HCO_3 (pH 7.9) at an enzyme–substrate ratio of 1:10 for periods of 24 to 72 hr at 37°. Two peptide fragments (MP-1 and MP-2) were purified by molecular sieve chromatography on P-6 Bio-Gel as described. Other peptide fragments were partially purified on G-50 Sephadex (superfine) equilibrated with 0.2 *M* NH_4HCO_3 (pH 8.2) in a column 145 × 1.2 cm. One such peptide fragment (MP-3) was fully purified on DEAE-Sephadex A-25, equilibrated with 0.1 *M* ammonium acetate (pH 5.0) in a column 5 × 0.5 cm with a gradient to 0.5 *M* ammonium acetate (pH 5.0). Another peptide (MP-4) was partially purified by chromatography on DEAE-Sephadex as described, and was finally purified on QAE-Sephadex A-25 equilibrated with 0.05 *M* NH_4HCO_3 (pH 9.4) in a column 5 × 0.5 cm with a gradient to 0.5 *M* NH_4HCO_3 (pH 9.4). The yield of staphylococcal protease peptides ranged between 40 and 50%.

Detection of Peptide Peaks. Column effluents were monitored for uv absorbance at 260 and 280 nm. Fluorescamine (Fluoram) was used to detect peptides by the method of Böhlen et al. (1973), using a fluoromicrophotometer (Aminco) to measure fluorescence at 390 nm.

C-Terminal Analysis with Carboxypeptidase. Carboxypeptidase A (8 μg) or carboxypeptidase B (4 μg) or both were added to the polypeptide or peptide fragments in 0.2 *M* $NaHCO_3$ (pH 8.2) for 30 to 180 min. After digestion, samples were acidified to pH 3.0 using 6 *N* acetic acid and lyophilized. The lyophilizate was dissolved in 0.2 *M* sodium citrate (pH 2.2) and applied to the amino acid analyzer.

Results

The complete amino acid sequence of ubiquitin was determined by automated sequential analysis on the intact

[1] Abbreviations used are: Pth, phenylthiohydantoin; MT, tryptic peptide of maleated ubiquitin; MC, chymotryptic peptide of maleated ubiquitin; MP, staphylococcal protease peptide of maleated ubiquitin.

Table I: Amino Acid Analysis of Native Ubiquitin and Maleated Tryptic Peptides of Ubiquitin

	Native	MT-1	MT-2	MT-3	MT-4	T1-dMT-2
Asp	7.16 (7)	3.4 (4)	2.2 (2)	1.0 (1)	0.0 (0)	0.2 (0)
Thr	6.71 (7)	4.6 (5)	2.4 (2)	0.1 (0)	0.1 (0)	0.9 (1)
Ser	3.25 (3)	0.7 (1)	2.2 (2)	0.1 (0)	0.1 (0)	0.9 (1)
Glu	12.21 (12)	8.4 (8)	2.4 (2)	2.2 (2)	0.0 (0)	1.3 (1)
Pro	3.40 (3)	3.3 (3)	0.3 (0)	0.1 (0)	0.0 (0)	0.0 (0)
Gly	4.28 (4)	2.1 (2)	0.3 (0)	2.2 (2)	0.1 (0)	0.0 (0)
Ala	2.15 (2)	1.2 (1)	0.2 (0)	1.1 (1)	0.0 (0)	0.0 (0)
Half-Cys	0.06 (0)	0.1 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Val	3.96 (4)	2.8 (3)	1.3 (1)	0.1 (0)	0.0 (0)	1.0 (1)
Met	0.92 (1)	0.8 (1)	0.1 (0)	0.1 (0)	0.0 (0)	0.0 (0)
Ile	6.70 (7)	4.6 (5)	1.3 (1)	1.0 (1)	0.0 (0)	0.1 (0)
Leu	9.18 (9)	2.5 (2)	4.2 (4)	2.3 (2)	1.0 (1)	3.2 (3)
Tyr	0.96 (1)	0.1 (0)	0.9 (1)	0.1 (0)	0.0 (0)	0.1 (0)
Phe	2.00 (2)	1.0 (1)	0.1 (0)	1.0 (1)	0.0 (0)	0.0 (0)
Lys	6.80 (7)	4.6 (5)	1.2 (1)	1.1 (1)	0.0 (0)	0.1 (0)
His	0.96 (1)	0.3 (0)	0.8 (1)	0.2 (0)	0.0 (0)	0.9 (1)
Arg	4.10 (4)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)
N-Terminus	Met	—	Thr	Leu	Leu	Glu
Total	74	42	18	12	2	9

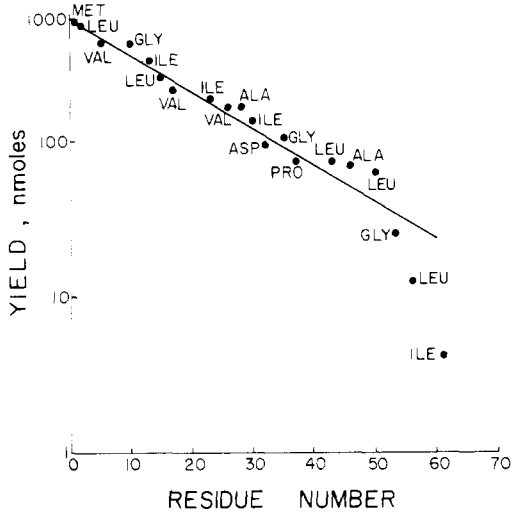

FIGURE 1: Yields of phenylthiohydantoin amino acids obtained during automated degradation of ubiquitin showing the repetitive yield through the first 50 steps.

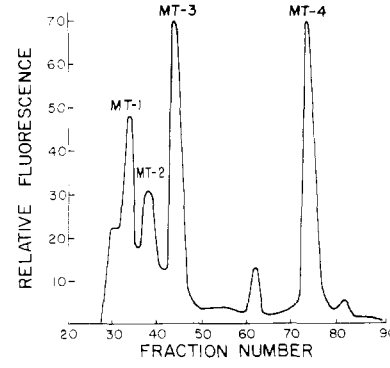

FIGURE 2: Elution profile of the tryptic digest of maleated ubiquitin on P-6 Bio-Gel chromatography. Four major peptide-containing peaks were detected by fluorescence after reaction with fluorescamine at pH 6.85. These are designated MT-1, MT-2, MT-3, and MT-4.

polypeptide and manual Edman degradations of maleated tryptic (MT) peptides. Since these provided minimal proof of primary structure, these data are presented first. Supporting and corroborative sequence analysis furnished by purified chymotryptic peptides of maleated ubiquitin (MC) and staphylococcal protease peptides of maleated ubiquitin (MP) is also presented.

Sequenator Analysis of Intact Ubiquitin. Batches of ubiquitin used were pure by the criteria of end-group analysis and polyacrylamide gel disc electrophoresis at pH 8.9 and 4.3 (Goldstein et al., 1975).

An automated sequence analysis of 1.0 μM ubiquitin is illustrated in Figure 1, which shows the yields of Pth amino acids at selected steps of the degradation. Positive identifications were made at each step from residue 1 through residue 61. The average repetitive yield at each step through 50 cycles was 94%.

Sequence of Maleated Tryptic Peptides. The elution profile of the maleated tryptic peptides on P-6 Bio-Gel is shown in Figure 2. Four major peptide fragments were obtained. The N-terminal analysis and amino acid compositions of intact ubiquitin and the four maleated tryptic peptides of ubiquitin are presented in Table I. Peptide MT-1, a maleated tryptic peptide consisting of 42 amino acids by amino acid analysis, possessed an amino terminus resistant to Edman degradation; it was taken to be the N-terminal peptide in ubiquitin with a maleated α-amino group and was not studied further. Peptide MT-2, consisting of 18 amino acids by amino acid analysis and possessing an N-terminal threonine, was degraded manually with positive identifications at each step through the first ten residues. The single lysine residue in peptide MT-2 was identified at position 9. Peptide MT-3 containing 12 amino acids with a C-terminal arginine was sequenced manually to the penultimate C-terminal residue. The C-terminal residue, arginine, was identified by carboxypeptidase digestion. Peptide MT-4 was sequenced manually and found to be Leu-Arg.

Sequence of Tryptic Peptide T1-dMT-2. The elution profile of the tryptic digest of demaleated peptide MT-2 on DEAE-Sephadex is shown in Figure 3. Amino-terminal and amino acid analysis data for peptide T1-dMT-2 are presented in Table I. Inasmuch as peptide T2-dMT-2, the second eluting peptide, possesses the same N-terminal residue (threonine) as intact MT-2, it was not studied further. The earlier eluting peptide, T1-dMT-2, possessed an N-terminal glutamic acid, which is the residue found in peptide MT-2

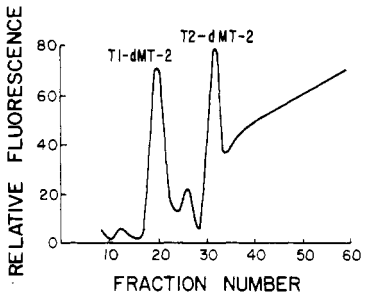

FIGURE 3: Elution profile of the tryptic digest of demaleated MT-2 on DEAE-Sephadex A-25. Two major peptide-containing peaks were detected by fluorescence after reaction with fluorescamine at pH 6.85. These are designated T1-dMT-2 and T2-dMT-2.

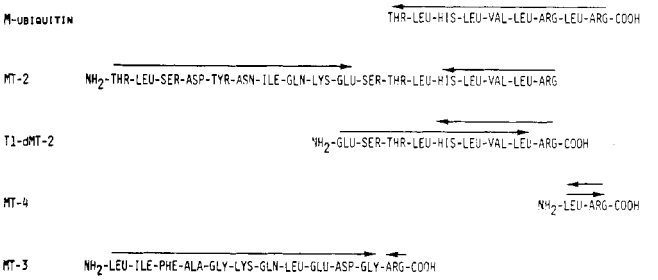

FIGURE 4: Amino acid sequence of peptides MT-2, MT-3, MT-4, and T1-dMT-2. Arrow to the right indicated residues identified by Edman degradation. Arrows to the left indicate residues identified by carboxypeptidase digestions. The C-terminal sequence of ubiquitin derived from carboxypeptidase analysis of maleated ubiquitin is indicated by M-ubiquitin. This sequence is consistent with placement of peptide MT-4 following peptide MT-2.

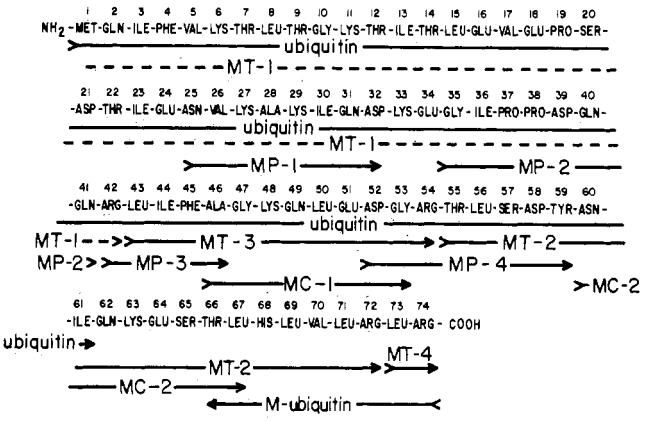

FIGURE 5: Complete amino acid sequence of bovine ubiquitin. Arrows to the right indicate that residues were identified by Edman degradations; they show the residues identified, not the total length of the peptide. Arrows to the left indicate residues identified by carboxypeptidase digestion. Dotted lines indicate placement of peptide MT-1 (see text). MT indicates maleated tryptic peptides, MP indicates maleated protease peptides, and MC indicates maleated chymotryptic peptides. MT peptides are ordered by size, not by placement in the sequence. The sequence through 61 residues was obtained from automated sequence analysis of intact ubiquitin.

following the lysine residue at position 9. This peptide was therefore considered to be C terminal in MT-2 and was degraded manually through the penultimate residue, with positive identifications at each step. C-Terminal analysis of T1-dMT-2 revealed the presence of Arg, Leu, Val, and His in molar ratios of 1.0:2.05:0.9:0.5, respectively, after a 3-hr digestion, which is consistent with the C-terminal pentapeptide sequence of peptide T1-dMT-2, -His-Leu-Val-Leu-Arg. Figure 4 summarizes the sequence analysis of peptides MT-2, T1-dMT-2, MT-3, and MT-4.

C-Terminal Analysis of Ubiquitin. Digestion of native ubiquitin with carboxypeptidase A alone resulted in the liberation of no free amino acid. Arginine was released during the digestion of ubiquitin with carboxypeptidase B alone, and a 30-min digestion of ubiquitin with carboxypeptidase A and B together resulted in the release of leucine and arginine in equal amounts, indicating that the C-terminal dipeptide sequence was Leu-Arg. When maleated ubiquitin was digested with carboxypeptidase A and B for 3 hr arginine, leucine, valine, histidine, and threonine were released in the molar ratios 2.0:4.0:0.9:0.8:0.4, respectively. These ratios are consistent with the placement of MT-4 (Leu-Arg) at the C terminus of ubiquitin, and provide support for a C-terminal nonapeptide sequence of Thr-Leu-His-Leu-Val-Leu-Arg-Leu-Arg (Figure 4).

Summary of Sequence Data Necessary for Minimal Proof of Primary Structure of Ubiquitin. Figure 5 presents the complete amino acid sequence of bovine ubiquitin. Residues 1 through 61 were obtained by automated sequence analysis of the intact molecule. Residues 43–54 and 55–64 were obtained by manual degradations on peptide MT-3 and MT-2. Residues 64 through 72 were obtained by manual degradations on peptide T1-dMT-2. Residues 73 and 74 were obtained from sequencing of peptide MT-4, and placement of this dipeptide at the C terminus was made by exclusion and, more positively, by C-terminal analysis of ubiquitin and maleated ubiquitin.

Supporting Data. Certain peptides were purified after digestion of maleated ubiquitin with chymotrypsin or staphylococcal protease and were sequenced manually. The sequences of the peptides are summarized in Figure 5; although they were eventually unnecessary to prove structure, they provide overlapping sequences confirming the data presented.

Discussion

Determination of the complete amino acid sequence of bovine ubiquitin was facilitated by an extremely long automated sequence determination that provided the majority of the sequence. It may be that the two arginine residues in the C-terminal tripeptide minimized extractive losses late in the degradation and thus permitted a high repetitive yield to be maintained. We were additionally fortunate that the four peptide fragments produced by tryptic digestion of maleated ubiquitin were fully resolved in one step by molecular sieve chromatography. Maleation was initially performed to mask the ϵ-amino groups of lysine residues and permit selective cleavage at the arginine residues. We found that native ubiquitin was resistant to digestion by trypsin, chymotrypsin, subtilisin, and carboxypeptidase, and that maleation served not only to limit the cleavage sites of trypsin but indeed to make the molecule susceptible to digestion by trypsin and other enzymes. The most likely interpretation of these findings is that ubiquitin has a tertiary structure dependent on internal bonding by lysine residues and that maleation induces a conformational change by preventing the formation of these bonds. Maleation may permit unfolding of the molecule and render more sites accessible to enzymatic digestion.

The several regions of internal sequence homology within ubiquitin (Figure 6) suggest that the coding gene arose by duplication of an antecedent gene, as proposed for other

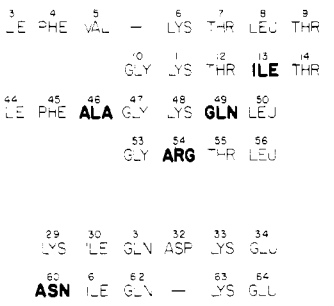

FIGURE 6: Portions of amino acid sequence of bovine ubiquitin aligned to show regions of internal sequence homology. Numbers denote the position at the residue in the sequence. Two deletions have been shown to improve the homology and five substitutions are shown by boldface type; these are all frequently seen substitutions (Dayhoff, 1972). The sequence -Gly-Lys-Thr-Leu- is seen, with modifications, four times.

proteins showing such internal homology (Dayhoff, 1972). The immunoglobulins are an excellent example of selective advantage conferred on vertebrates by such an evolutionary mechanism. The case of ubiquitin is unique, however, in that this protein is probably essential to all present-day organisms. Therefore, if the duplication hypothesis is correct, ubiquitin appeared at a very primitive stage of cellular evolution and conferred an advantage sufficient for the exclusion of all other competing contemporary life forms.

The structure of ubiquitin is of interest for several reasons. Firstly, this molecule exhibits extraordinary evolutionary conservation in cells of animals, yeast, bacteria, and higher plants, and shows close similarity by functional, immunological, and structural criteria when isolated from such diverse sources as bovine thymus and celery (Goldstein et al., 1975); ubiquitin clearly subserves some vital function in the living organism to have been conserved over this immense evolutionary time span. Secondly, ubiquitin activates adenylate cyclase in many tissues and appears to do so via β-adrenergic receptor, inasmuch as its effect is blocked by the β-adrenergic antagonist propranolol (Goldstein et al., 1975). Ubiquitin must therefore have a binding site that mimics the low molecular weight ligand epinephrine. It should be of great interest to determine the sequence of amino acid residues in ubiquitin that produce such a β-adrenomimetic binding site.

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