Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 9411–9415, December 1988
Biochemistry

Secretion in yeast of human lysozymes with different specific activities created by replacing valine-110 with proline by site-directed mutagenesis

(*Saccharomyces cerevisiae*/cis/trans isomerization/evolution)

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Communicated by Robert L. Letsinger, September 6, 1988

ABSTRACT **Computer graphics indicate that a steric hindrance exists between valine-110 side chain of human lysozyme (EC 3.2.1.17) and an acetyl group of a modified substrate that contains N^6,*O*-diacetylmuramic acid. To alter the substrate specificity of human lysozyme to be effective on the modified substrate, we replaced the valine-110 residue with various amino acids by site-directed mutagenesis. One of the mutant proteins (valine residue replaced with proline: P^{110}) was secreted in *Saccharomyces cerevisiae* as at least four components (P^{110}-A, P^{110}-B, P^{110}-C, and P^{110}-D) with different specific activities. Two components, P^{110}-B and P^{110}-D, were isolated in a pure form and structurally characterized. The results suggest that this mutation lowered the lytic activity against *Micrococcus lysodeikticus* by changing a local conformation of the catalytic site while keeping almost the same substrate binding sites. Our results also indicate that cis/trans isomerization of prolyl peptide bonds probably occurs *in vivo* and that the conformational change of protein as well as point mutations in genes might influence the molecular evolution of the protein.**

Human lysozyme (EC 3.2.1.17) (h-lysozyme), secreted in a native form by *Saccharomyces cerevisiae* using the chicken lysozyme (c-lysozyme) signal sequence (1, 2) and its modified derivatives (2, 3), is easily purified from the culture fluid and crystallized (2). This suggests that the same protein folding system is present in yeast as in mammalian cells. We reasoned, therefore, that the yeast secretion system is suitable for engineering h-lysozyme and analyzing the tertiary structures of the mutant proteins.

h-Lysozyme consists of 130 amino acid residues with four disulfide bonds. It acts specifically on the β-1,4-glycosidic linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine, which are the principal components of the cell walls of *Micrococcus lysodeikticus*. However, *Staphylococcus aureus* has modified cell walls, in which 60% of the hydroxyl groups at the 6 position of *N*-acetylmuramic acid are acetylated (4). Computer graphics indicate that there is a steric hindrance between the Val-110 side chain of h-lysozyme and an acetyl group of the substrate. This appears to be one of the reasons why h-lysozyme cannot act on the cell walls of *S. aureus*. To alter the substrate specificity to be effective on *S. aureus*, Val-110 residue was replaced with several amino acids that might admit the acetyl group into the cleft of the h-lysozyme. One of the mutant proteins thus obtained (Val-110 → Pro; P^{110}) was synthesized and secreted by *S. cerevisiae* as more than four components with different specific activities. This finding seems to contradict the theory "one gene and one enzyme." The results described here also suggest not only the existence of *in vivo* cis/trans isomerization of prolyl peptide bonds but also some influence on the molecular evolution of the protein.

To elucidate some of the features of the mutant h-lysozymes P^{110}, we describe their purification and characterization.

MATERIALS

Klenow fragment of DNA polymerase I and restriction enzymes were purchased from Boehringer Mannheim or Takara Shuzo (Kyoto, Japan), T4 DNA ligase was from New England Biolabs, h-lysozyme and *M. lysodeikticus* cells were from Sigma, *S. aureus* IFO 12712 was from Institute for Fermentation Osaka (Osaka, Japan), and glycolchitin and $(GlcNAc)_3$ were from Seikagaku Kogyo (Tokyo). Enzyme reactions were carried out under the conditions recommended by the suppliers.

METHODS

Strains and Media. *S. cerevisiae* AH22R⁻(a, *leu2, his4, can1, pho80*) (5) was used for the host strain and cultivated in modified Burkholder medium (6) supplemented with 8% sucrose.

DNA. Oligonucleotides were synthesized by the phosphoramidite method (7) on the Applied Biosystems (Foster City, CA) DNA Synthesizer (model 380A) and purified by HPLC on a TSK gel ODS-120T (Toyo Soda, Tokyo).

Oligonucleotide-Directed Mutagenesis. Plasmid pGEL125 (2), which contains the cloned yeast glyceraldehyde-3-phosphate dehydrogenase promoter and DNA encoding the chemically synthesized c-lysozyme signal sequence and h-lysozyme in that order, was used as a starting material for mutagenesis. For mutagenesis, the 0.5-kilobase *Xho* I fragment of DNA encoding h-lysozyme was cut out of pGEL125 and converted to the *Hin*dIII fragment with the aid of an *Xho* I/*Hin*dIII linker. The obtained fragment was subcloned into the *Hin*dIII site of M13mp18 to obtain M13LZM. Two synthesized oligonucleotides, 5′-TCGAGGCCA-3′ (9-mer) and 5′-AGCTTGGCC-3′ (9-mer), were used as an *Xho* I/*Hin*dIII linker. To replace Val-110 with proline, oligonucleotide-directed mutagenesis by the method of Zoller and Smith (8) was carried out using 5′-CCAAGCG*GG*CCAGGC-3′ (15-mer) as a primer.

Sequencing and Plasmid Construction. To confirm the mutation, a Takara M13 sequencing kit (Takara Shuzo) was

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Abbreviations: h-lysozyme, human lysozyme; c-lysozyme, chicken lysozyme.

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used for sequencing by the dideoxy method (9). The [α-^{32}P]dCTP was purchased from Amersham. The wild-type h-lysozyme gene on pGEL125 was replaced by the *Xho* I fragment of the mutated gene to obtain pGEL302P.

Determination of Lysozyme. The lysis of *M. lysodeikticus* cells (0.2 mg/ml) in 0.1 M potassium phosphate buffer (pH 6.2) was monitored at 450 nm. One unit of h-lysozyme was defined as the amount of protein that decreases 0.001 of A_{450} per min at 25°C. Hydrolysis of glycolchitin by h-lysozyme was determined by the method of Yamada and Imoto (10). The synthesized h-lysozyme was also detected by Western blotting using rabbit anti-h-lysozyme antibody (11) and horseradish peroxidase-conjugated goat anti-rabbit IgG (H + L) (Bio-Rad).

Determination of Dissociation Constants. The dissociation constants of h-lysozyme and its derivatives for $(GlcNAc)_3$ were determined in 0.1 M acetate buffer (pH 5.5) at 25°C by ultraviolet difference spectroscopy using a DU-40 spectrophotometer (Beckman). The protein concentrations were 3.4×10^{-5} M and the $(GlcNAc)_3$ concentrations varied from 2.0×10^{-5} M to 4.0×10^{-4} M. Based on the absorbance differences at 293 nm, the dissociation constants were calculated by an iterative computer program (Y.Y., unpublished data).

Measurement of NMR. Each sample for NMR measurement contained 0.5–1.0 mM protein in ^2H_2O. The pH was adjusted to 6.80 by the addition of NaO^2H solution. Proton magnetic resonance spectra were obtained with a JEOL GX 500s NMR spectrometer. The temperature dependence was measured from 30°C to 80°C with a step of 10°C.

Measurement of CD. CD was measured with a J-20 spectropolarimeter (Japan Spectroscopic, Tokyo) at 20°C. The proteins were dissolved in 0.1 M potassium phosphate buffer (pH 6.2) and the concentrations were adjusted to 0.5 mg/ml. The data were expressed in terms of mean residue ellipticity [θ]. The secondary structures were estimated by using the program of Provencher and Glöckner (12).

Determination of Amino Acid Composition. The purified protein (20 μg) was subjected to acid hydrolysis with 6 M HCl at 110°C for 24 hr and the amino acid composition was analyzed with a Hitachi 835 Amino Acid Analyzer.

Determination of N-Terminal Amino Acid Sequence. The N-terminal amino acid sequence of purified protein was determined by Edman degradation in a pulse-liquid phase protein sequencer model 477A (Applied Biosystems).

Determination of SH Group. SH group was determined with Ellman's reagent (13). The procedure followed that of Honda *et al.* (14).

NaDodSO$_4$/PAGE. NaDodSO$_4$/PAGE was carried out by the method of Laemmli (15) using 15% polyacrylamide gels in the presence of 0.1% NaDodSO$_4$. When the electrophoresis was performed under reducing conditions, the sample was treated with 5% 2-mercaptoethanol at 100°C for 5 min.

RESULTS

Analysis of Mutant h-Lysozyme P^{110}. No lytic activity against *M. lysodeikticus* and *S. aureus* was observed in the culture fluid of *S. cerevisiae* harboring pGEL302P, which encodes the mutant h-lysozyme P^{110}. As indicated in Fig. 1, the gene products in the 3-day culture were analyzed by Western blotting. The secreted mutant h-lysozyme P^{110} exhibited the same mobility as the native h-lysozyme molecule on NaDodSO$_4$/PAGE under reducing conditions. However, mutant P^{110} gave two bands under nonreducing conditions on NaDodSO$_4$/PAGE. One band migrated slower than the native enzyme and the other exhibited the same mobility. This suggests that the heterogeneous proteins were synthesized from the same gene.

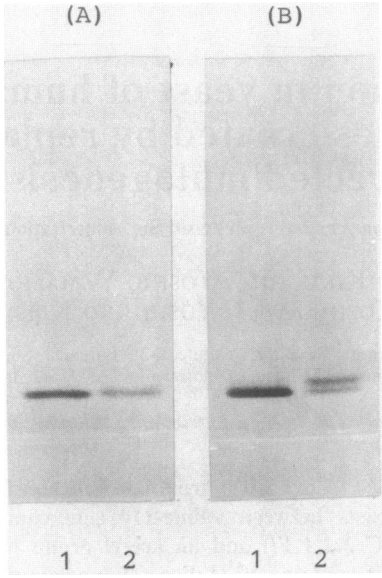

FIG. 1. Western blot analysis of the culture supernatants. NaDodSO$_4$/PAGE of 3-day culture supernatants was carried out as described. The proteins were transferred to a nitrocellulose filter (Schleicher & Schuell, BA85) and detected by Western blotting. (*A*) Reducing conditions. (*B*) Nonreducing conditions. Lanes: 1, the supernatant of *S. cerevisiae* AH22R$^-$/pGEL125 (native h-lysozyme); 2, the supernatant of *S. cerevisiae* AH22R$^-$/pGEL302P (mutant h-lysozyme P^{110}).

Purification of Mutant h-Lysozyme P^{110}. To characterize this mutant h-lysozyme P^{110}, *S. cerevisiae* AH22R$^-$ harboring pGEL302P was grown at 28°C for 72 hr. The culture supernatant (40 liters) was applied to a CM-Toyopearl 650C (Toyo Soda) column (4 × 34 cm) equilibrated with 50 mM phosphate buffer (pH 6.0). After the column was washed with 2 liters of the same buffer, the adsorbed protein was eluted with the buffer containing 0.5 M NaCl. Only one peak (480 ml) was obtained. This fraction was further purified by cation exchange HPLC (Asahipak ES-502CP). The mutant protein was eluted with a linear gradient of Na_2SO_4 as shown in Fig. 2. Mutant P^{110} was separated into at least four peaks designated P^{110}-A, P^{110}-B, P^{110}-C, and P^{110}-D. All components were isolated in a pure form and subjected to the experiments described below. However, the amounts of P^{110}-A and P^{110}-C were so small that only the specific activities were determined.

Substrate Specificity and Dissociation Constant. None of the proteins demonstrated any activity against *S. aureus*. Conversion of Val-110 with proline lowered the enzymic activity against *M. lysodeikticus* as follows: P^{110}-A, 0.11%; P^{110}-B, 0.24%; P^{110}-C, 6.0%; P^{110}-D, 2.9% that of the native enzyme (Table 1). Proteins P^{110}-B and P^{110}-D showed 4.5% and 13.1% of the activity of the native enzyme against glycolchitin as a substrate. However, the dissociation constant of the mutants P^{110}-B and P^{110}-D and the native enzyme for $(GlcNAc)_3$ were almost the same. These results indicate that Val-110 contributes to the conformation for retaining enzymic activity but not for substrate binding; that is, some local conformation was changed around the catalytic site.

Amino Acid Composition and N-Terminal Amino Acid Sequence. The N-terminal amino acid sequence of the purified P^{110}-B and P^{110}-D was a single sequence, N-Lys-Val-Phe-Glu-Arg-Xaa-Glu-Leu-Ala-Arg, that was identical to the authentic h-lysozyme. To confirm the amino acid substitution, a sample each of P^{110}-B and P^{110}-D was subjected to amino acid analysis (data not shown) and the mutant proteins showed the expected change (Val → Pro). In the mutant proteins, the number of valine residues was reduced by one

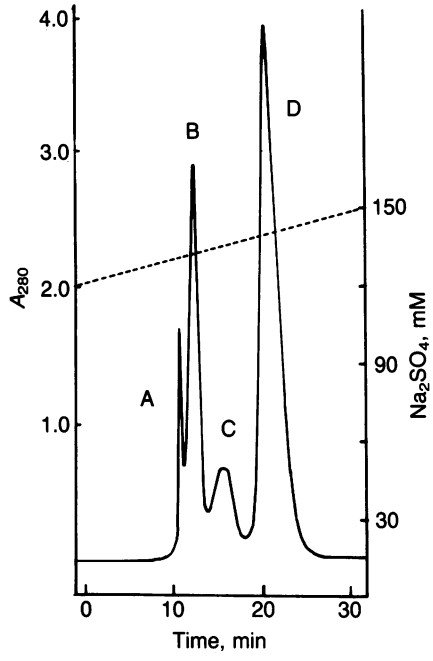

FIG. 2. HPLC of the mutant h-lysozyme P^{110} contained in the eluate from CM-Toyopearl column. The eluate from CM-Toyopearl column was dialyzed against distilled water and a 15-ml portion was injected directly into the Asahipak ES-502CP (2.15 × 10 cm). A 5-ml/min linear gradient of 0.6 M Na_2SO_4 in 50 mM phosphate buffer (pH 6.5) divided P^{110} into four peaks. A, P^{110}-A; B, P^{110}-B; C, P^{110}-C; D, P^{110}-D.

and the number of proline residues was increased by one. The number of other amino acids was the same as that of the native enzyme. However, P^{110}-B differed from P^{110}-D and the native enzyme by possessing 0.88 mol of SH group in its molecule (see *Discussion*).

NMR Spectra. Fig. 3 shows the temperature dependence of the NMR spectra of h-lysozyme, P^{110}-B, and P^{110}-D. The spectrum of P^{110}-D obtained at 50°C resembled that of h-lysozyme obtained at the same temperature. In particular, the spectral pattern was quite similar in the region of 5.0–6.2 ppm, where the signals of C(α) protons were observed. These C(α) proton signals observed in such a low magnetic field result from the ring current effect of the aromatic residues nearby. Therefore, the spectral resemblance suggests that the local structures in the vicinity of C(α) protons are conserved between h-lysozyme and P^{110}-D. Fewer signals were observed with P^{110}-B in this spectral region. h-Lysozyme held the structure even at 70°C, and the spectrum changed to the pattern typical of the random coil state at 80°C. The thermal stability of P^{110}-D was as high as that of h-lysozyme. However, the structure of P^{110}-B was more unstable and changed into the random coil structure below 60°C. These results indicate that P^{110}-D has a structure similar to that of h-lysozyme, whereas the structure of P^{110}-B is different and is thermally unstable.

Table 1. Specific activities and dissociation constants of native and mutant h-lysozymes

h-Lysozyme	Enzymic activity, % Against *M. lysodiekticus*	Against glycolchitin	K_d for $(GlcNAc)_3$, M
Native	100	100	3.6×10^{-5}
P^{110}-A	0.11	ND	ND
P^{110}-B	0.24	4.5	3.8×10^{-5}
P^{110}-C	6.0	ND	ND
P^{110}-D	2.9	13.1	2.7×10^{-5}

ND, not determined.

CD Spectra. To study conformational changes of the proteins in solution, CD spectra of P^{110}-B, P^{110}-D, and the native enzyme were obtained (Fig. 4). The spectrum of P^{110}-B was a little different from that of the other two, but all were considered to be essentially identical. The secondary structures of the mutant proteins were also estimated according to the method of Provencher and Glöckner (12) (Table 2). The α-helix content of the native enzyme, P^{110}-B, and P^{110}-D was estimated to be 30%, 31%, and 29%, and their β-sheet content

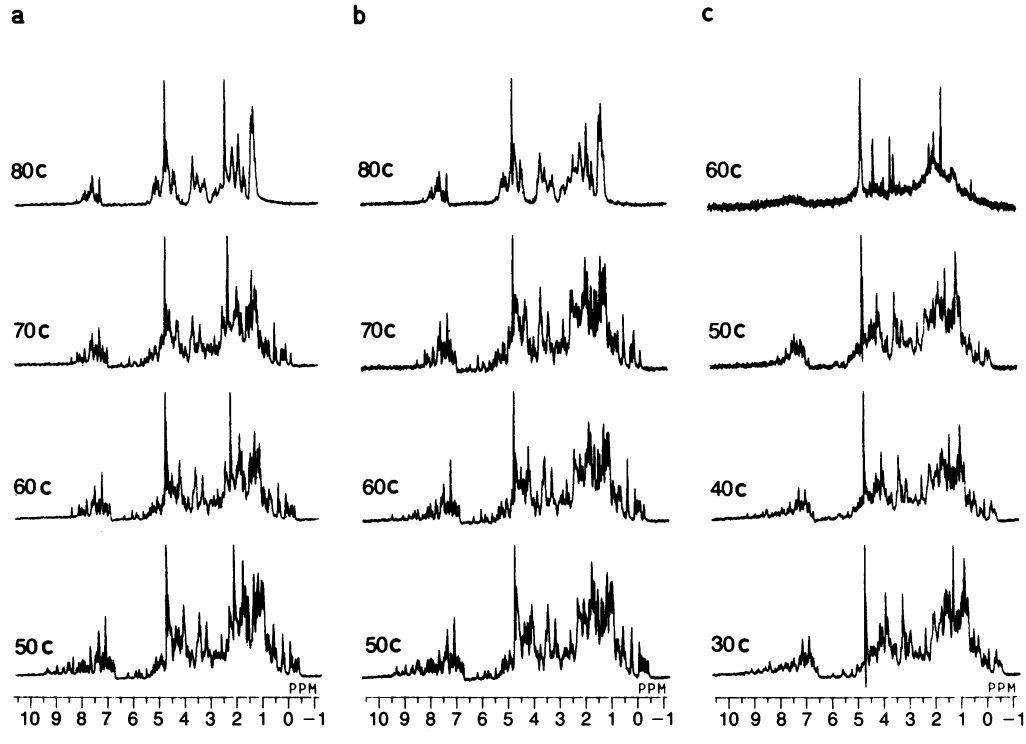

FIG. 3. Proton NMR spectra of h-lysozyme (*a*), P^{110}-D (*b*), and P^{110}-B (*c*) at various temperatures.

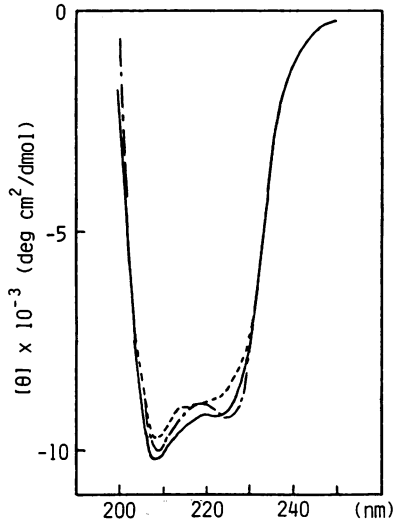

FIG. 4. CD spectra of h-lysozyme (—), P^{110}-B (---), and P^{110}-D (-·-).

was estimated to be 18%, 19%, and 20%, respectively. The α-helix and β-sheet content of the native h-lysozyme were calculated to be 34% and 15% based on the x-ray data (16). These are in good agreement with the values calculated by the method of Provencher and Glöckner (12). It is reasonable to conclude that the native enzyme and the mutants P^{110}-B and P^{110}-D have almost the same conformation. Some conformational change certainly has occurred but conformational differences were not detected on CD spectra.

DISCUSSION

It is generally believed that one gene directs the synthesis of one protein. However, the replacement of Val-110 of h-lysozyme with proline by site-directed mutagenesis in yeast gave several immunoreactive proteins with the same primary structure but different local conformations. Our previous results suggested the presence of the same protein folding system in yeast and mammalian cells (2), because h-lysozyme is secreted in a native form by *S. cerevisiae*. In this system, DNA encoding the native h-lysozyme gives one homogeneous protein; however, a gene encoding mutant h-lysozyme P^{110}(Val-110 → Pro) gave several proteins with different specific activities. One plausible explanation would be that cis/trans isomerization of proline has triggered the synthesis of heterogeneous proteins in yeast.

Recently, isomerization in protein folding has attracted considerable attention (17). Evans *et al.* (18) reported in experiments *in vitro* using staphylococcal nuclease that cis/trans isomerism might cause the conformational multiplicity. The other refolding experiments of denatured proteins *in vitro* by Lang *et al.* (19) pointed out that prolyl peptide bond isomerization is the rate-determining step in protein folding. These results suggest that the isomerization of propyl peptide bonds is not only a process in protein folding, but the interconverting state between unfolded and folded proteins. However, not much is known about the isomerization of prolyl peptide bonds in protein folding *in vivo*.

Table 2. Estimation of secondary structures of h-lysozyme, P^{110}-B, and P^{110}-D

h-Lysozyme	α-Helix, %	β-Sheet, %
Native	30 (34)	18 (15)
P^{110}-B	31	19
P^{110}-D	29	20

The secondary structures were estimated by using the program of Provencher and Glöckner (12). The α-helix and β-sheet contents of the native h-lysozyme were calculated based on the x-ray data (16) and are indicated in parentheses.

Isolated mutant proteins P^{110}-B and P^{110}-D showed almost the same characteristics in enzymic activity, dissociation constant for $(GlcNAc)_3$, amino acid composition, and N-terminal amino acid sequence, but differed strikingly in that P^{110}-B has a free SH group and is more unstable than P^{110}-D. These features may indicate that conformations of P^{110}-B and P^{110}-D are considerably different. There exists the possibility that one cysteine in P^{110}-B is modified in some fashion, causing thermal instability. Human leukocyte interferon A produced in *Escherichia coli* is modified by glutathione at the thiol group of cysteine residues (20). Analysis of the amino acid composition indicates that no change occurred in P^{110}-B and P^{110}-D except at proline and valine. This seems to demonstrate that no modification of the cysteine residue by glutathione had occurred. Other modifications may have occurred at the cysteine residue and further examination is needed. The CD spectra suggested that P^{110}-B and P^{110}-D have almost the same conformations. This contradiction might be explained by assuming that the conformational change after residue 110, if it occurred, does not appear on the CD spectrum because residue 110 is located near the C terminus of the molecule. More detailed examination will be necessary, but the replacement of valine with proline certainly caused conformational heterogeneity of mutant h-lysozyme proteins. Isolated P^{110}-B and P^{110}-D have been crystallized and the crystal form of the latter is the same as that of the native h-lysozyme, but that of the former is completely different. Detailed information will be reported elsewhere.

The remaining question is why so many proteins are produced by replacing Val-110 with proline. This question has not yet been answered, but we speculate that the introduction of proline at residue 110 may affect two other proline residues at positions 71 and 103, which are present in the h-lysozyme molecule. Factors influencing protein folding *in vivo* may be a proline residue itself and additional unknown environments such as the local conformation around a proline residue.

Our results also indicate the importance of residue 110 of h-lysozyme for enzymic activity. Replacement of Val-110 with glycine as well as proline decreased the enzymic activity of h-lysozyme (data not shown) but did not alter the dissociation constant for $(GlcNAc)_3$, an inhibitor of h-lysozyme. These results indicate that residue 110 plays an important role in determining the local conformation of the catalytic site but not that of the substrate binding site. The x-ray analysis of c-lysozyme (21), whose primary structure is very similar to that of h-lysozyme, revealed that c-lysozyme has six substrate binding sites (A, B, C, D, E, and F) in its molecule. The preferential binding sites are A, B, and C, and the catalytic site exists between D and E. Another x-ray analysis of h-lysozyme confirmed that the tertiary structures of these two lysozymes are very similar (16). These results suggest that the binding sites in h-lysozyme are the same as in c-lysozyme. Based on this assumption, Val-110 is located near the D site so that it might be reasonable that the replacement of Val-110 with other amino acids does not affect the binding of $(GlcNAc)_3$ to sites A, B, and C but does affect the catalytic site.

This phenomenon of a single gene producing several active forms of a protein might influence protein evolution. The discussion on protein evolution so far has concerned point mutations in genes encoding specific proteins and mutations affecting the rate of synthesis of specific proteins. Our findings described here have indicated that proteins with different specific activities are synthesized from the same gene. Such difference in activities might involve some type of

possible regulation of the level of enzyme; however, further data are necessary to assess the significance of our finding.

We thank Drs. Y. Sugino and A. Kakinuma for providing the plasmid pGEL125; Drs. Y. Nakao, K. Nara, and K. Kitano for culturing *S. cerevisiae* AH22R$^-$/pGEL302P; and Drs. K. Nakahama and R. Marumoto for helpful advice and discussion.

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