Protein Mobility and Self-Association by Deuterium Nuclear Magnetic Resonance†

Jan B. Wooten and Jack S. Cohen*

ABSTRACT: Hen egg white lysozyme has been prepared in which the C^{ϵ} position of the single histidine residue is substituted by a deuterium atom as a nondisturbing stable isotope probe. The deuterium nuclear magnetic resonance (^2H NMR) spectrum in H_2O shows a broad resonance (500–1000 Hz) due to the histidine deuteron and a sharp signal from residual HOD. The line width of the deuterium signal increases with pH, reflecting the self-association of lysozyme which is known to involve this histidine [Shindo, H., Cohen, J. S., & Rupley, J. A. (1977) *Biochemistry 16*, 3879]. Correlation times calculated from spin–spin relaxation times (T_2) derived from the ^2H line widths indicate that His-15 is restricted in motion and that lysozyme is predominantly dimerized at pH 7.5. Controls carried out with [ϵ-^2H]imidazole showed a small pH dependence of the spin–lattice relaxation time (T_1), which parallels the ^2H chemical shift change upon ionization of the imidazole. Similar results cannot generally be observed by proton nuclear magnetic resonance (^1H NMR) because of paramagnetic relaxation due to trace metal ion impurities. The pH dependence of the ^2H T_1 values indicates a change in the ^2H quadrupole coupling constant upon protonation of the imidazole ring.

Spectroscopic studies of magnetic nuclei in biological systems have generally concentrated on those nuclei which provide the highest possible spectral resolution. Deuterium, because of its nuclear quadrupole moment, exhibits broad nuclear resonances in slowly reorienting molecules and hence does not lend itself to high-resolution NMR studies. However, the particular properties of the deuteron can be taken advantage of in other respects; the intrinsic nuclear relaxation due to the quadrupole moment renders the deuteron an effective monitor of local mobility independent of other nuclei. This property has been extensively used to study deuterated membrane systems (Seelig, 1977), the binding to proteins of small molecules (Szilagyi et al., 1977; Andrasko & Forsen, 1974; Gerig & Rimerman, 1972; Zens et al., 1976), and selectively deuterated peptides (Glasel et al., 1973), but there have been no direct applications to proteins. One reason for this fact is that the broadness of deuterium NMR signals precludes the resolution of multiple signals. Therefore, to obtain meaningful results from the application of this method to proteins it is necessary to deal with a singly deuterium-enriched molecule. We now report the first such study of mobility in a protein, hen egg white lysozyme, containing a single deuterium atom. Previously, only heme methyl groups of myoglobin have been enriched with deuterium (Oster et al., 1975). Lysozyme was chosen principally for two reasons: first, it contains a single histidine residue at position 15, and this could be selectively deuterated (Meadows et al., 1968); and second, it self-associates as a function of pH in a manner in which the histidine residue is known to be involved (Shindo et al., 1977). Thus, this system provides a convenient model for the utility of deuterium NMR as a probe of macromolecular mobility.

Experimental Procedures

Materials. Hen egg white lysozyme (Worthington Biochemical Corp.) was exchanged at pH 9 in 99.7% D_2O at 37 °C in a sealed tube under nitrogen. The substitution of the C^{ϵ} proton of His-15 with ^2H was monitored by the disappearance of the ^1H resonance in the 220-MHz proton NMR spectrum. After 1 week, the solution was neutralized with 1 N DCl, lyophilized, and then lyophilized 3 times from ^2H-depleted water (Aldrich) to remove residual D_2O. The [ϵ-^2H]imidazole was prepared under similar conditions.

^2H NMR Measurements. The 41.4-MHz ^2H spectra of 11 and 7 mM solutions of lysozyme in 0.2 N NaCl were obtained at 25 °C on a home-built spectrometer equipped with a Bruker superconducting magnet. Typically, 20 000 scans were obtained by using a spectra window of 20 kHz. A 10-Hz exponential line broadening was applied before Fourier transformation. A 180°–τ–90° pulse sequence was used to partially suppress the sharp interfering resonance due to residual HOD, with τ chosen such that the much broader lysozyme resonance was not disturbed. The line widths at half-height ($W_{1/2}$) were obtained by fitting the observed spectra with Lorentzian lines using the Curve Analysis Program on the Nicolet 1180 data system. The ^2H T_1 values of 0.5 M solutions of [ϵ-^2H]-imidazole were measured by using the standard inversion–recovery pulse sequence. The ^2H chemical shifts were measured relative to the ^2H resonance of CD_3CN (1%) in the aqueous solutions.

Results

[ϵ-^2H]Imidazole. The ^2H NMR chemical shift of [ϵ-^2H]imidazole is essentially the same as the ^1H shift of the normal imidazole, and the pK_a value obtained, 6.960 ± 0.007 (Figure 1), agrees with that obtained from ^1H NMR spectra, 7.15 (Sachs et al., 1971). Addition of fairly high levels of paramagnetic Cu^{2+} ion (10^{-4} M) results in an additional pH inflection, which presumably reflects the formation of Cu–Im[1] complex (Sundberg & Martin, 1974). The ^2H relaxation time, T_1, also reflects the ionization of the imidazole ring with an apparent pK_a = 6.69 ± 0.09 (Figure 2). In the presence of Cu^{2+}, the T_1 titration curve is somewhat altered, but the ^2H resonance is readily observable even at this high Cu^{2+} ion concentration.

[ϵ-^2H]His-15 Lysozyme. A typical ^2H spectrum is shown in Figure 3 and consists of two resonances, that of the singly ^2H-labeled protein and the residual HOD. Since there is such a difference in the line width between these signals, no dif-

† From the Developmental Pharmacology Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20205. *Received May 2, 1979.* This work was presented at the 20th Experimental NMR Spectroscopy Conference, Asilomar, California, Feb 1979.

[1] Abbreviations used: Im, imidazole; tri-NAG, tri-*N*-acetylglucosamine.

This article not subject to U.S. Copyright. Published 1979 by the American Chemical Society

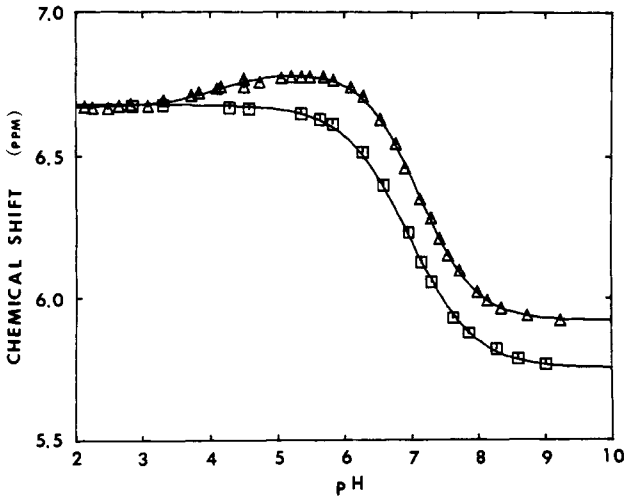

FIGURE 1: ^2H chemical shift relative to CD_3CN as a function of pH for [ϵ-^2H]imidazole. (□) 0.5 M imidazole; (△) 0.5 imidazole + 10^{-4} M Cu^{2+}.

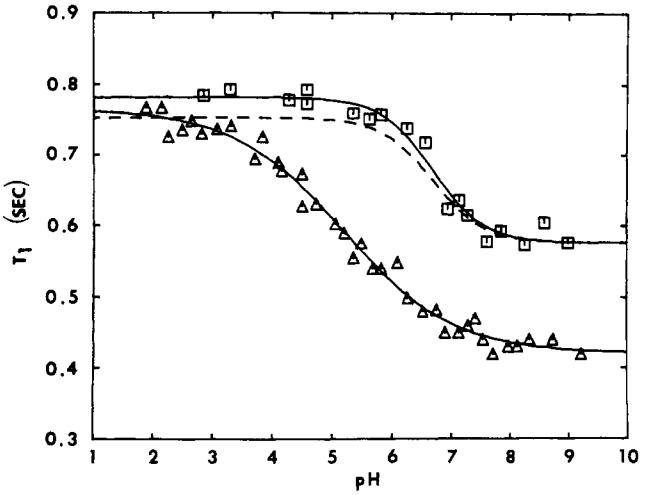

FIGURE 2: ^2H spin–lattice relaxation times of [ϵ-^2H]imidazole as a function of pH. (□) 0.5 M imidazole; (△) 0.5 imidazole + 10^{-4} M Cu^{2+}. (—) Best fit; (- - -) predicted for a change of Q_D from 168 kHz (pH <6.8) to 192 kHz (pH >6.8).

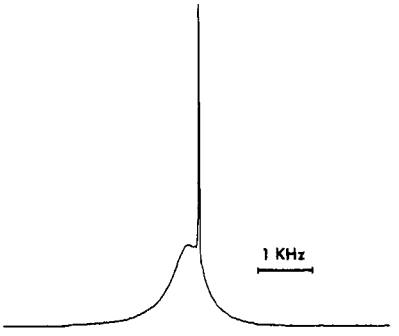

FIGURE 3: 41.4-MHz ^2H spectrum of [ϵ-^2H]His-15 lysozyme (11 mM) in 0.2 M NaCl at pH 3.0.

ficulty was experienced in curve-fitting the spectra with a sum of two Lorentzian peaks. The line widths for the deuteron resonance are plotted in Figure 4 as a function of pH. At the higher lysozyme concentration studied (11 mM) the line widths were consistently greater at any pH value than at the lower concentration (7 mM), indicating a greater degree of self-association. Upon addition of the inhibitor tri-NAG, the line width was significantly reduced even at pH 4.5, suggesting a significant degree of self-association even at pH <4.5.

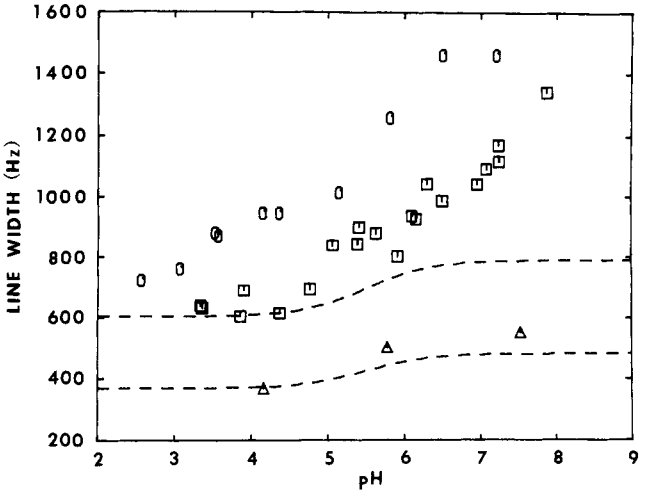

FIGURE 4: ^2H line widths (hertz) for [ϵ-^2H]His-15 lysozyme as a function of pH. (○) 11 mM lysozyme; (□) 7 mM lysozyme; (△) 7 mM lysozyme + tri-NAG. Dotted lines indicate line widths for τ_c = 5.7 ns (lower) and τ_c = 13.3 ns (upper).

Table I: Correlation Times for [ϵ-^2H]His-15 Lysozyme from ^2H NMR Measurements at 41.4 MHz and 25 °C

	pH	$W_{1/2}$ (Hz)	Q_D (kHz)	τ_c (ns)	$\frac{r_a}{r_m}$ [a]
11 mM lysozyme	4.2	947	168	22.6	1.59
	7.2	1464	192	27.1	1.69
7 mM lysozyme	4.3	605	168	13.3	1.33
	7.3	1172	192	21.3	1.55
7 mM lysozyme + tri-NAG	4.2	360	168	5.65	1.00
	7.5	543	192	7.52	1.10

[a] Radius of the aggregate relative to the monomer. The monomer line width was taken as the line width of 7 mM lysozyme in the presence of tri-NAG at pH 4.2.

Correlation times (τ_c) were calculated from the relationship (Abragam, 1961)

$$W_{1/2} = 1/\pi T_2 = \frac{3\pi}{10} Q_D^2 \left(1.5\tau_c + \frac{2.5\tau_c}{1 + \omega^2\tau_c} + \frac{\tau_c}{1 + 4\omega^2\tau_c^2} \right) \quad (1)$$

where $W_{1/2}$ is the line width at half-height, T_2 is the spin–spin relaxation time, and ω is the resonance frequency in angular units (Table I). The choice of the deuteron quadrupolar coupling constant, Q_D, depended on the ionization state of the imidazole ring (see Discussion). The mean radius of lysozyme in solution as calculated from the well-known Stokes–Einstein relationship (Abragam, 1961) increased by a factor of ~1.7 on raising the pH from 4.5 to 7.5, indicating that dimerization is predominant at higher pH.

Discussion

Although the ^2H line width can be quite broad in slowly reorienting molecules, it remains sensitive to small changes in the correlation time (Figure 5). In order to estimate the line width, however, it is necessary to employ a single specific ^2H substitution, since broad overlapped resonances cannot in general be resolved. We have accomplished this for lysozyme by exchanging protons for deuterons at high pH followed by acidification and exchange in H_2O. Under these conditions only the C^ϵ position of the imidazole ring of His-15 does not exchange back. Hence, we have prepared lysozyme labeled with a single deuterium atom.

The caveat of Wasylishen & Cohen (1974) that proton T_1 values of C^ϵ protons in protein histidines may be dominated

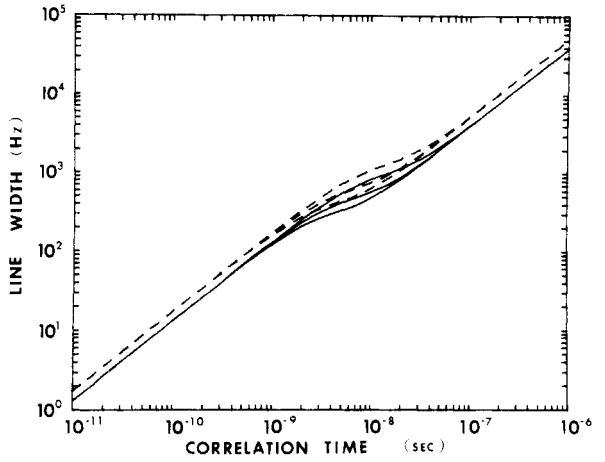

FIGURE 5: ^2H line width vs. reorientational correlation time (τ_c) for isotropic reorientation at 13.8, 27.6, and 41.4 MHz (top to bottom) for Q_D = 168 kHz (—) and Q_D = 192 kHz (- - -).

by paramagnetic relaxation due to trace metal ions suggests that similar precautions should be taken for deuteron relaxation. The relaxation of deuterons, however, is dominated by quadrupolar relaxation (Mantsch et al., 1977; Johnson & Everett, 1972), and trace paramagnetic ions should not significantly affect deuteron relaxation times. Nonetheless, a clear inflection was observed in the pH titration curve of the T_1 value for [ϵ-^{2}H]imidazole (Figure 2). The addition of relatively high amounts of paramagnetic Cu^{2+} ion caused the inflection to become more shallow and the T_1 values to be somewhat depressed as the pH was increased. There is ample evidence, however, that this behavior results from an increase in the effective correlation time of imidazole due to complexation with Cu^{2+} (Wooten et al., 1975, 1976) rather than to the paramagnetic interaction. The indirect coupling constant between C^ϵ and its adjoining proton ($^1J_{CH}$) in imidazole also exhibits a pH dependence (Wasylishen & Tomlinson, 1975). Since both $^1J_{CH}$ and Q_D are known to depend on the carbon hybridization (Mantsch et al., 1977), it appears reasonable that the inflection in the T_1 titration curve results from a change in Q_D (and thus also in the relaxation rate) upon ionization of the imidazole. While a change in correlation time might result from a difference in solvation of the protonated and nonprotonated imidazole species, a difference in Q_D is more likely. Using the best available estimates of Q_D for a carbon with sp^2 hybridization for neutral imidazole, 192 kHz (Diehl & Reinhold, 1978), and with sp^3 hybridization for charged imidazole, 168 kHz (Wooten et al., 1979), we obtained a nearly perfect simulation of the pH dependence of the T_1 (Figure 2). Therefore, we feel this is the more likely basis for the observed titration of T_1. It should be noted that since the relaxation of ^2H is so much less sensitive than that of ^1H to paramagnetic metal ions, the chemical shifts and T_1 values (Figures 1 and 2) could be measured at Cu^{2+} concentrations at least one order of magnitude greater than would be possible to observe broadened ^1H resonances of imidazole. Indeed, the great sensitivity of T_1 values of the C^ϵ proton to trace amounts of paramagnetic metal ion (down to 10^{-6} M) obviates the use of such evaluations for proteins (Wasylishen & Cohen, 1974). The broad Lorentzian peak arising from [ϵ-^{2}H]His-15 lysozyme (Figure 3) showed a significant pH dependence (Figure 4). This was greater than expected on the basis of the characteristic changes in Q_D found to be appropriate for [ϵ-^{2}H]imidazole itself.

From the increase in line width from low to high pH it is clear that the mean correlation time of the deuteron is increasing, particularly above pH 4.5. From eq 1 values of the correlation time were calculated at low and high pH values (Table I). Addition of the inhibitor tri-NAG at high pH led to a significant decrease in the ^2H line width, to the value of lysozyme alone at pH <4.5. It is known that tri-NAG causes disaggregation of lysozyme (Sophianopoulous, 1969) and that His-15 is involved in the head-to-tail self-association process at high pH (Shindo et al., 1977). It is noteworthy that at low pH with tri-NAG the line width is even less than that of lysozyme alone, indicating some degree of self-association even under these conditions. From the lowest value of the line width obtained for the presumed monomer by comparison with the values of the line width at pH 7.5, a ratio of the molecular radii of 1.7 is obtained from the Stokes–Einstein relation. This indicates that the protein is on the average predominantly dimerized at pH 7.5. This is consistent with the results of other techniques (Sophianopoulous & Van Holde, 1961, 1964; Deonier & Williams, 1970).

It is noteworthy that the correlation time for the histidine residue as determined from the deuterium signal at low pH is the same as the value (13 ns) found for the α carbons from ^{13}C NMR studies under the same experimental conditions (Wilbur et al., 1976). Since the α carbons reflect the overall backbone correlation time, this implies that the His-15 residue has little local freedom of rotation and that it is rigidly held to the protein molecule. Consideration of the structure determined by X-ray crystallography (Blake et al., 1965) confirms this conclusion. A space-filling model of the protein generated by computer indicates that the histidine residue is relatively buried. In addition, a standard ball and stick model allows one to see clearly the two hydrogen bonds connecting the imidazole ring to other groups in the molecule (Figure 6). These hydrogen bonds would undoubtedly restrict the motion of this residue. This result is also consistent with the low pK_a value (5.3) of His-15 determined from proton NMR studies (Shindo et al., 1977), which indicates that the imidazole ring is relatively unavailable for protonation.

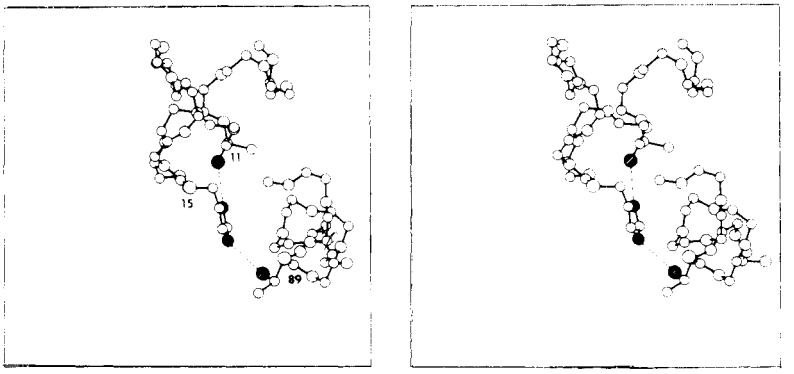

FIGURE 6: Hydrogen bonds to His-15 from residues Thr-89 and Ala-11 from the X-ray crystallographic structure.

We have described the simplest case of a single ^2H label in a single histidine residue in a protein. Due to the known limited resolution of proton NMR signals of multiple resonances of a given type of residue in a protein, such as histidines at high and low pH values and tyrosine or methionine residues, one can predict that deuterium resonances of the same residues will not be resolved, particularly in view of the deuterium line widths. However, deuterium labeling and deuterium NMR observation may be a convenient general method for measuring protein mobility and self-association in solution. We are extending our studies to other systems where the deuterium probe may supply unique information.

References

Abragam, A. (1961) *The Principles of Nuclear Magnetism*, Oxford University Press, London.
Andrasko, J., & Forsen, S. (1974) *Chem. Scr. 6*, 163.
Blake, C. C. F., Koenig, D. F., Mair, G. A., North, A. C. T., Phillips, D. C., & Sarma, V. R. (1965) *Nature (London) 206*, 757.
Deonier, R. C., & Williams, J. W. (1970) *Biochemistry 9*, 4260.
Diehl, P., & Reinhold, M. (1978) *Mol. Phys. 36*, 143.
Gerig, J. T., & Rimerman, R. A. (1972) *J. Am. Chem. Soc. 94*, 7565.
Glasel, J. A., Hruby, V. J., McKelvy, J. F., & Spatola, A. F. (1973) *J. Mol. Biol. 79*, 555.
Johnson, A., & Everett, G. W., Jr. (1972) *J. Am. Chem. Soc. 94*, 1419.
Mantsch, H. H., Saito, H., & Smith, I. C. P. (1977) *Prog. Nucl. Magn. Res., Spectrosc. 11*, 211.
Meadows, D. H., Jardetsky, O., Epand, R. M., Ruterjans, H. H., & Scheraga, H. H. (1968) *Proc. Natl. Acad. Sci. U.S.A. 60*, 766.
Oster, O., Neireiter, G. W., Clouse, A. O., & Gurd, F. R. N. (1975) *J. Biol. Chem. 250*, 7990.
Sachs, D. H., Schechter, A. N., & Cohen, J. S. (1971) *J. Biol. Chem. 246*, 6576.
Seelig, J. (1977) *Q. Rev. Biophys. 10*, 353.
Shindo, H., Cohen, J. S., & Rupley, J. A. (1977) *Biochemistry 16*, 3879.
Sophianopoulous, A. J. (1969) *J. Biol. Chem. 244*, 3188.
Sophianopoulous, A. J., & Van Holde, K. E. (1961) *J. Biol. Chem. 236*, PC82.
Sophianopoulous, A. J., & Van Holde, K. E. (1964) *J. Biol. Chem. 239*, 2516.
Sundberg, R. J., & Martin, R. B. (1974) *Chem. Rev. 74*, 471.
Szilagyi, L., Harangi, J., & Radics, L. (1977) *Biophys. Chem. 6*, 201.
Wasylishen, R. E., & Cohen, J. S. (1974) *Nature (London) 249*, 847.
Wasylishen, R. E., & Tomlinson, G. (1975) *Biochem. J. 147*, 605.
Wasylishen, R. E., & Cohen, J. S. (1977) *J. Am. Chem. Soc. 99*, 2480.
Wilbur, D. J., Norton, R. S., Clouse, A. O., Addleman, R., & Allerhand, A. (1976) *J. Am. Chem. Soc. 98*, 8250.
Wooten, J., Savitsky, G. B., & Jacobus, J. (1975) *J. Am. Chem. Soc. 97*, 5027.
Wooten, J. B., Beyerlein, A. L., Jacobus, J., & Savitsky, G. B. (1976) *J. Am. Chem. Soc. 98*, 6490.
Wooten, J. B., Savitsky, G. B., Jacobus, J., Beyerlein, A. L., & Emsley, J. W. (1979) *J. Chem. Phys. 70*, 438.
Zens, A. P., Fogle, P. T., Bryson, T. A., Dunlap, R. B., Fisher, R. R., & Ellis, P. D. (1976) *J. Am. Chem. Soc. 98*, 3760.

Isolation and Characterization of Fourteen Ribosomal Proteins from Small Subunits of Yeast†

Ken-ichi Higo and Eiko Otaka*

ABSTRACT: A method for preparation of a large amount of ribosomal subunits from *Saccharomyces cerevisiae* by a Ti-15 zonal rotor is described. The proteins of the small subunits (ca. 50 000 A_{260} units) were separated into 22 fractions by chromatography on carboxymethylcellulose columns. Fourteen proteins were then purified from the ten chromatographic fractions by filtration through Sephadex G-100 or Sephacryl S-200. The isolated proteins are YP 6, YP 7, YP 9, YP 12, YP 14′, YP 14″, YP 28, YP 38, YP 45, YP 50, YP 52, YP 58, YP 63, and YP 70. The molecular weights and amino acid compositions of these proteins are presented.

In a previous paper (Otaka & Kobata, 1978), we identified 74 proteins from yeast ribosomes by two-dimensional polyacrylamide gel electrophoresis. A large-scale preparation of these individual ribosomal proteins has become necessary for sequencing studies. Here, we describe, first, a procedure for preparing a large amount of small and large subunits from yeast cells. The ribosomal proteins prepared from the respective subunits were then fractionated by carboxymethylcellulose (CMC)[1] column chromatography. Most of the protein fractions from the first CMC column chromatography contained more than one protein species and were therefore further fractionated by chromatography on columns of Sephadex G-75, Sephadex G-100, Sephacryl S-200, or phosphocellulose. As a result, we have so far succeeded in purifying 14 ribosomal proteins from small subunits and 23 from large subunits in milligram quantity. Recently, one of

† From the Department of Biochemistry and Biophysics, Research Institute for Nuclear Medicine and Biology, Hiroshima University, 2-3, Kasumi 1 chome, Hiroshima, 734 Japan. *Received April 20, 1979.* This work was supported by Grant-in-Aid for Scientific Research from the Ministry of Education of Japan (No. 348375) awarded to S. Osawa. This paper is the second in a series entitled Yeast Ribosomal Proteins. For the preceding paper, see Otaka & Kobata (1978).

[1] Abbreviations used: CMC, carboxymethylcellulose; TMD-I buffer, 50 mM Tris-HCl (pH 7.4)–10 mM $MgCl_2$–1 mM dithiothreitol; DTT, dithiothreitol; TD buffer, 50 mM Tris-HCl (pH 7.4)–1 mM dithiothreitol; TMD-II buffer, 50 mM Tris-HCl (pH 7.4)–0.1 mM $MgCl_2$–1 mM dithiothreitol; 2-D electrophoresis, two-dimensional electrophoresis; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/79/0418-4191$01.00/0 © 1979 American Chemical Society