storage for 1 to 2 weeks at 4 °C gave a chromatographic pattern from Sepharose 6B identical with that of a freshly prepared sample.

In addition to the size heterogeneity, the conjugates formed are heterogeneous in their sites and numbers of disulfide bond linkages. This is inevitable as the conversion of lysyl residues of proteins A or B into reactive thiol or mixed disulfide groups occurs randomly to give a mixture of protein derivatives differing in their sites and numbers of lysyl residues modified. Therefore, on coupling such mixtures of proteins A and B, different pairings of disulfide bonds will result. This type of heterogeneity can be avoided if specific modifications of a single site of proteins A or B can be carried out, but this is difficult to attain.

The protein conjugates prepared in this work have molar ratios of the two proteins of about 1.5 as compared with the expected value of one. This is shown for the conjugate of albumin with D-GL on amino acid analysis and for the conjugate of specific sheep antibody and horseradish peroxidase on enzyme assay and antigen binding activity. The retention of biological activities of the conjugates, their good yields, and ease of preparation all indicate the usefulness of the procedure described in this paper.

Acknowledgment

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Pressure-Induced Changes in the Nuclear Magnetic Resonance Spectra of a Biopolymer in Aqueous Solution†

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ABSTRACT: High resolution proton nuclear magnetic resonance spectra of the poly(amino acid) poly[N^5-(3-hydroxypropyl)-L-glutamine], of degrees of polymerization 685 and 137, were measured in a mixed D_2O and H_2O solvent, at pressures from 1.03 to 1968.5 kg/cm^2, and at temperatures of 2 and 10 °C. Increasing the pressure appeared to cause an increased mobility of the side chain hydrocarbon residues, and also of the α-hydrocarbon residue of the polymer chain. This is interpreted to imply the occurrence of a volume decrease on unfolding of the polymer from a helix to a random coil, with subsequent exposure of hydrophobic groups to the solvent.

The study of the denaturation of proteins by pressure has been the source of considerable recent interest due to the difficulties which have occurred in interpreting the results (Brandts et al., 1970; Zipp & Kauzmann, 1973; Li et al., 1976; Williams & Shen, 1972). Attempts to explain these results in terms of model compound studies have not been completely successful (Kliman, 1969).

In order to bridge this gap, we have studied the effect of pressure on molecules of high molecular weight but relatively simple structure, namely poly(amino acids) (Suzuki & Tani-

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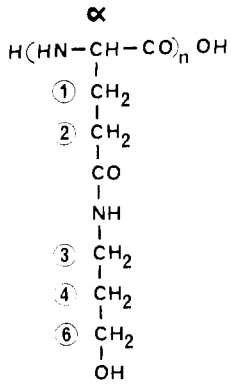

FIGURE 1: Structure of the polymer poly[N^5-(3-hydroxypropyl)-L-glutamine]. Peaks are numbered according to position in the polymer as shown in this figure. Symbols: d = DSS peak; w = water band.

guchi, 1968, 1967; Gunter & Gunter, 1972). In addition we have used the technique of high resolution nuclear magnetic resonance spectroscopy to monitor the changes. To date, no studies of the effect of pressure on biopolymers have been made by this method, other than a preliminary study of the present system (Williams et al., 1978).

The polymer chosen for this study was poly[N^5-(3-hydroxypropyl)-L-glutamine] (PHPG),[1] which can be synthesized in various chain lengths (Lup-Lotan et al., 1965, 1966) and which has a side chain with several hydrophobic residues arranged in sequence. The polymer is known to exist both in the helical and the random coil forms and to undergo a reversible equilibrium between them. It has no ionizable group in its structure and so the helix–coil transition is not dependent on, or obscured by, the ionization of such a group, but should depend to a great extent on the effect of hydrophobic groups.

Experimental Procedure

Materials. Poly(γ-benzyl-L-glutamate) of degree of polymerization 685, or 137, was obtained from Sigma Chemicals. 3-Amino-1-propanol (J. T. Baker Chemical Co.) was dried over barium oxide and redistilled at atmospheric pressure. 1,4-Dioxane (J. T. Baker) was purified over sodium hydroxide, filtered, dried over sodium, and redistilled at atmospheric pressure. Deuterium oxide, 99.75% D (J. T. Baker), was used as supplied. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as supplied (Stohler Isotopes, Waltham, Mass.).

Methods. The polymer poly[N^5-(3-hydroxypropyl)-L-glutamine], of the required degree of polymerization, was synthesized by the method described by Lup-Lotan et al. (1965) from poly(γ-benzyl-L-glutamate) and 3-amino-1-propanol in 1,4-dioxane as solvent.

High pressure glass capillaries of inner diameter ca. 1 mm and outer diameter ca. 4–4.5 mm were prepared from Pyrex glass tubing as will be described separately (Williams, 1978; Yamada, 1974). They were connected by high pressure stainless steel tubing to a conventional high pressure pump (American Instrument Co., Silver Spring, Md).

The temperature of the samples were adjusted by means of a standard Varian Control Unit. Nitrogen gas, precooled by liquid nitrogen, and heated by the temperature regulator, was blown over the glass capillary to thermostat the latter. It was found that the NMR spectra observed did not change appreciably with time after 1 min of equilibration, when the temperature was changed by 10 °C, or the pressure adjusted. Therefore spectra were observed after 5-min equilibration.

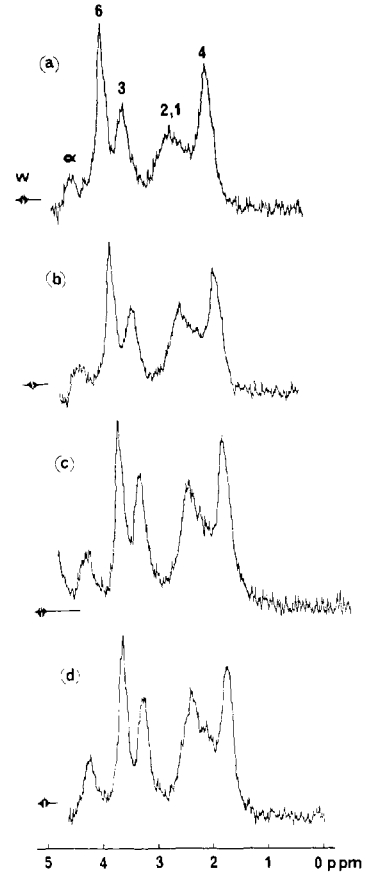

FIGURE 2: Spectra of the polymer PHPG, DP 685, in a D_2O–H_2O mixture, at a temperature of 2 °C, and pressures in kg/cm² of (a) 1.03, (b) 703.05, (c) 1406.1, (d) 1968.5.

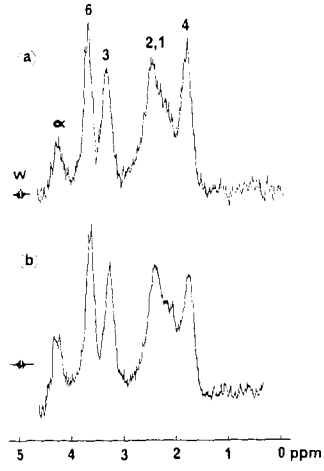

FIGURE 3: Spectra of the polymer PHPG, DP 685, in a D_2O–H_2O mixture, at a temperature of 10 °C, and pressure in kg/cm² of (a) 1.03, (b) 1968.5.

Solutions of the polymer were made by dissolving a 10% (w/v) proportion of the freeze-dried polymer in a 10:1 (v/v) mixture of D_2O and H_2O. For chemical shift measurements an internal standard of 1% (w/v) sodium 2,2-dimethyl-2-silapentane-5-sulfonate, DSS, was added to the solution. Spectra are shown with a scale referred to the DSS proton resonance peak.

Polymer residues were numbered according to the num-

[1] Abbreviations used: PHPG, poly[N^5-(3-hydroxypropyl)-L-glutamine]; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

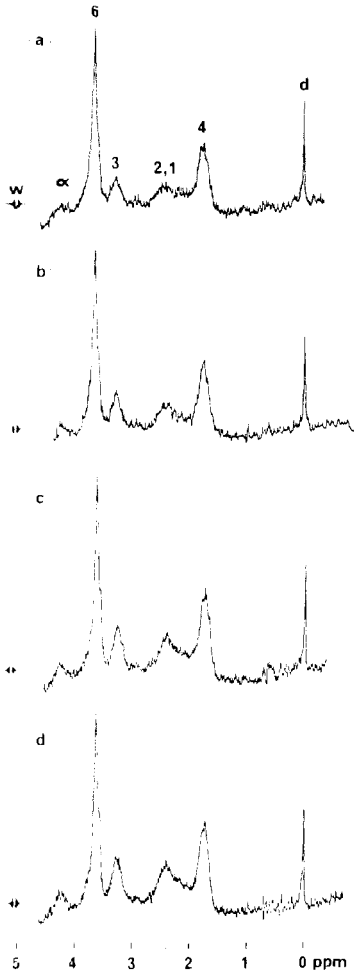

FIGURE 4: Spectra of the polymer PHPG, DP 137, in a D_2O-H_2O mixture, containing DSS at a temperature of 2 °C, and at pressures in kg/cm^2 (a) 1.03, (b) 351.5, (c) 1406.1, (d) 1968.5.

bering convention used by Joubert et al. (1970) as seen in Figure 1. The assignment of proton resonance peaks used by these authors was also adopted (Figures 2–4).

Spectra were recorded on a Varian H.A. 100 spectrometer with 15-in. magnet and commercial V 4333 probe. The water band of the D_2O and H_2O mixture was used as lock signal.

Determination of the helix content of the polymers was obtained as follows. Circular dichroism spectra were recorded in a Cary 61 spectropolarimeter, in a mixture of D_2O and H_2O, in a 10:1 (v/v) ratio, at a concentration of approximately 1 mg/mL (w/v). The mean residue ellipticity $[\theta]$ was calculated, based on a dry weight concentration. The helix content was calculated according to the method of Greenfield & Fasman (1969) and also according to the method of Chen & Yang (1971).

Results

In Figures 2 and 3 are shown the NMR spectra of the polymer PHPG, of degree of polymerization 685, at temperatures of 2 and 10 °C, and at various pressures from 1.033 to 1968.5 kg/cm^2. On reducing the pressures from its highest value, the 1-atm spectrum obtained was subsequently substantially the same as obtained before pressurising (Williams et al., 1978).

Figure 4 shows the nuclear magnetic resonance spectra at lower degree of polymerization, 137, which is one-fifth of the size of the longer chain polymer. The spectra were recorded at 2 °C and at various pressures. It was again found that the effect of pressure was reversible and that on releasing the pressure from its highest value the original 1-atm spectrum was substantially recovered.

TABLE I: Values of the Mean Residue Ellipticities, $[\theta]_{222}$ and $[\theta]_{208}$, for the Polymer Poly[N^5-(3-hydroxypropyl)-L-glutamine] in a D_2O-H_2O Mixture.

Polymer	Concn (mg/mL)	Temp (°C)	$-[\theta]_{222} \times 10^{-3}$ [a]	$-[\theta]_{208} \times 10^{-3}$ [b]	% helix (λ 222)	% helix (λ 208)
PHPG (DP 685)	0.81	0	13 296	13 908	36	34
	0.81	25.2	7 064	9 638	16	19
	0.81	51.5	3 673	6 907	4	10
PHPG (DP 137)	0.86	0	15 406	15 317	43	39
	0.86	25.4	7 570	10 142	17	21
	0.86	51.5	3 903	6 949	5	10

[a] Calculated by the method of Greenfield & Fasman (1969). [b] Calculated by the method of Chen & Yang (1971).

In Table I are shown values of the mean residue ellipticities, $[\theta]_{222}$ and $[\theta]_{208}$, calculated as described by Greenfield & Fasman (1969), and by Chen & Yang (1971) from the circular dichroism spectra of the polymer poly[N^5-(3-hydroxypropyl)-L-glutamine], in a D_2O-H_2O mixture of ratio 10:1 (v/v).

Discussion

The NMR spectra obtained in Figures 2, 3, and 4 at any one temperature and degree of polymerization of the polymer show that the proton resonance peaks of the side chain CH_2 residues 1, 2, and 3 closest to the helix increased in sharpness and size with increasing pressure. In comparison the proton resonance peak of the terminal CH_2, residue number 6, of the side chain, did not change appreciably with increase in pressure. That this was true was confirmed by comparing the proton resonance peak of the $CH_2(6)$ residue to that of DSS, as is seen in Figure 4. In addition from Figures 2–4, it can be seen that the sharpness and size of the proton resonance peak of the α(CH) residue also increase with increasing pressure relative to that for the $CH_2(6)$ residue. The effects are relatively small but they are consistent and in the same direction in all cases. Similar results were obtained repeatedly.

Comparison of the results obtained for the polymer at the longer and shorter chain lengths shows that the same pressure dependence was obtained qualitatively in both cases. However, the pressure dependence at the lowest temperature, of 2 °C, seemed generally to be greater. At higher temperatures, between 30 and 50 °C, there was little effect.

It is of interest that the properties of liquid water such as compressibility and shear viscosity are affected by pressure. Both the viscosity and compressibility of liquid water behave anomalously in the pressure range 1–3 kbar, especially at temperatures between 0 and 20 °C. There may be structural changes which could affect the interaction of the protein with water (Eisenberg & Kauzmann, 1969).

From the results in Table I, it is seen that the percent helix of the polymer is higher at lower temperatures.

If these effects were due to a change in rotational diffusion time of the polymer molecule as a whole with pressure, then it would be expected that all the proton resonance peaks corresponding to the side chain groups and the α(CH) residue would be equally affected (Joubert et al., 1970). This is not the case.

Thus one explanation for the effect of pressure on the NMR spectra of a polymer of particular chain length is that an increased mobility of the side chain CH_2 residues closest to the helix occurs together with an increased mobility of the α(CH) residue on the helix. There is relatively little effect on the outermost CH_2 residue of the side chain which is much more mobile under all conditions. The sensitivity of the polymer to pressure is greatest when the polymer is tightly coiled, which is the case at lower temperatures, and less when it is loose, which is the case at higher temperatures. Since the random coil form of the polymer would allow greater mobility of the side chains, it would appear that increased pressure favors an uncoiling of the helix, as does increase in temperature, and that, in keeping with le Chatelier's principle, this uncoiling process must be accompanied by a decrease in volume.

Our results, based on a study of a molecule of large molecular weight comparable to that of a protein, thus tend to confirm the direction of change expected from the work on 4-octanone (Kliman, 1969) and to indicate a negative volume change on unfolding of the polymer from a coiled up structure to an unfolded one, with subsequent exposure of hydrophobic groups to the solvent.

The results clearly indicate the feasibility of investigating the pressure dependent properties of biopolymers by high resolution NMR spectroscopy, this being the first such study reported.

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Purification and Some Properties of the Histidyl-tRNA Synthetase from the Cytosol of Rabbit Reticulocytes†

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ABSTRACT: The histidyl-tRNA synthetase of rabbit reticulocyte cytosol has been purified 84 000-fold to apparent homogeneity with a specific activity of 687 nmol of histidyl-tRNA formed per min per mg of protein. Ten to 15% of the enzyme activity is sedimented with the ribosomes while the remainder is in the cytosol. The purified enzyme has a molecular weight of 122 000 as determined by sucrose density gradient centrifugation. Gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate suggests that it is composed of two similar subunits with a molecular weight of approximately 64 000. The enzyme has a magnesium optimum of 45 mM; however, this is reduced to 5 mM in the presence of an intracellular potassium concentration (160 mM). The enzyme acylates the two histidine tRNA isoacceptors of rabbit reticulocytes with similar K_m values and at similar rates.

The aminoacyl-tRNA synthetases play a critical role in translating the genetic code because they alone possess the double specificity to recognize both amino acid and tRNA. The His-tRNA[1] synthetase has been purified from *Salmonella typhimurium* (DeLorenzo & Ames, 1970), *Escherichia coli* (Kalousek & Konigsberg, 1974), and *Saccharomyces cerevesiae* (Boguslawski et al., 1974), and the enzyme from each source has been partially characterized. Purification and characterization of this enzyme from animal cells have not, however, been reported previously.

The His-tRNA synthetase has been of particular interest in our investigation of the translational apparatus of rabbit red cell precursors and in the function of the $tRNA^{His}$ isoaccepting species in hemoglobin synthesis (Smith, 1975). Hemoglobin contains relatively more histidine than other proteins, and $tRNA^{His}$ is enriched in reticulocytes as an aspect of special-

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[1] Abbreviations used: $tRNA^{His}$, histidine tRNA; $tRNA^{His}_1$ and $tRNA^{His}_2$ are isoaccepting histidine tRNA species which are eluted from reversed phase chromatographic columns in the order indicated; His-tRNA, histidyl-tRNA; $NaDodSO_4$, sodium dodecyl sulfate.

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