Proton Nuclear Magnetic Resonance Study of an Active Pentapeptide Fragment of Ubiquitin†

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ABSTRACT: The aqueous solution conformation of Tyr-Asn-Ile-Gln-Lys (UB5) corresponding to positions 59–63 of the polypeptide, ubiquitin, has been investigated by proton NMR. Like the parent protein, UB5 induces nonspecifically both T and B lymphocyte differentiation. The various NH and CH resonances of this pentapeptide have been assigned, and its solution conformation has been probed through a study of chemical shift variations with pH, temperature dependence of amide hydrogen chemical shifts, vicinal NH–$C^{\alpha}H$ and $C^{\alpha}H$–$C^{\beta}H_2$ coupling constant data, and amide hydrogen-exchange rates. The latter were measured in H_2O by using a combination of transfer of solvent saturation and saturation recovery NMR experiments. The data are compatible with the assumption of a highly motile dynamic equilibrium among different conformations for this peptide. The various secondary amide hydrogens remain essentially exposed to the solvent. The temperature-dependence study of the amide hydrogen chemical shifts also did not reveal any strong internal hydrogen bonds. A rotamer population analysis of tyrosine and asparagine side chains suggests that two of the rotomers are predominantly populated for each of these residues. From these results, a picture emerges of the dynamic conformation of UB5 in aqueous solution.

Ubiquitin is a 74 amino acid polypeptide which was first isolated from bovine thymus during isolation of the thymic polypeptide hormone thymopoietin (Goldstein et al., 1975). This polypeptide, which exhibits an extraordinary conservation of its primary structure, is found to be present not only in the thymus and other human tissue extracts but also in such widely diverse sources as animal cells, yeast, bacteria, and higher plants (Goldstein et al., 1975). Ubiquitin has been shown to be a component of chromatin protein A24 (Goldknopf & Busch, 1977; Hunt & Dayhoff, 1977; Kuehl et al., 1980; Matsui et al., 1979). It can also occur as a free polypeptide in trout testis chromatin (Watson et al., 1978). The biosyntheses of ubiquitin by parathyroid gland (Hamilton & Rouse, 1980) and in rat brain hypothalamus and human pituitary tissue (Seidah et al., 1978; Scherrer et al., 1978) have also been reported. More recently, ubiquitin has been identified to be the heat-stable ATP-dependent proteolysis factor 1 (Wilkinson et al., 1980), which is one of the components essential for protein degradation in reticulocytes (Hershko et al., 1979). One of the properties of this protein, whose primary biological function and evolutionary origin are currently being explored in several laboratories, is that it induces nonspecific differentiation of both T and B lymphocytes in vitro via β-adrenergic receptors and adenylate cyclase activation (Gershwin et al., 1979; Goldstein et al., 1975; Kagan et al., 1979; Scheid et al., 1978).

The amino acid sequence of this 8451-dalton polypeptide has been determined (Schlesinger et al., 1975). It was found that the amino acid sequence of human ubiquitin was identical with that from bovine thymus (Schlesinger & Goldstein, 1975). A preliminary crystallographic investigation of ubiquitin has been reported recently (Cook et al., 1979). Acid and thermal denaturation studies using ^1H NMR[1] indicated an exceptionally stable structure for this protein (Lenkinski et al., 1977). A spectroscopic investigation of ubiquitin by circular dichroism, ultraviolet absorbance, and fluorescence methods revealed that the secondary structure of this stable protein contains only a low percentage of α helix or β sheet (Jenson et al., 1980). Two synthetic fragments have been shown to retain the cell-differentiating activity of the parent protein: first a hexadecapeptide corresponding to positions 59–74 (Schlesinger et al., 1978) and a pentapeptide corresponding to positions 59–63 (Jenson et al., 1980). Thus, the synthetic pentapeptide Tyr-Asn-Ile-Gln-Lys (UB5) appears to correspond to the biologically active site of the parent ubiquitin molecule. It is, therefore, of interest to define the conformation–activity relationship in this pentapeptide, which is more amenable to study by physicochemical methods. Toward this end, in the present investigation, we have employed various NMR techniques to characterize the free solution conformation of UB5.

Materials and Methods

(*1*) *Materials and Sample Preparation.* The pentapeptide, UB5, was prepared by solid-phase synthesis (Jenson et al., 1980). Deuterium oxide (D_2O, 99.8% D), concentrated DCl,

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[1] Abbreviations used: NMR, nuclear magnetic resonance; UB5, ubiquitin$_{59-63}$; TSP, sodium 2,2,3,3-tetradeuterio-3-(trimethylsilyl)-propionate.

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and NaOD (both of 99.5% D) were purchased from Stohler Isotope Chemicals (Waltham, MA).

A sample concentration of ~30 mM in D_2O or H_2O was used in the proton NMR studies. The sample pH was varied by adding appropriate amounts of DCl (HCl) or NaOD (NaOH). All the studies were performed in 5 mm o.d. standard NMR sample tubes (Wilmad Glass Co., Inc., Buena, NJ). The sample pH values were measured by an Ingold combination glass electrode, Model 6030-02, and reported as direct pH meter readings without correction for the deuterium isotope effects. This is reasonable since the isotope effects on the electrode and the pK_a values nearly cancel (Bundi & Wuthrich, 1979).

(*2*) *NMR Studies.* Proton NMR spectra of UB5 in D_2O were recorded on a Bruker HX-270 (270-MHz) spectrometer operating in the Fourier transform (FT) mode. For samples in H_2O, proton NMR experiments were performed on the Brandeis University 270-MHz NMR spectrometer operating in the 2–1–4 pulse mode (Redfield et al., 1975; Redfield, 1978) and on the Carnegie-Mellon University 250-MHz NMR spectrometer operating in the correlation mode (Dadok & Sprecher, 1974; Gupta et al., 1974). Additional ^1H NMR measurements including two-dimensional NMR experiments were performed on a Bruker WH-400 (400-MHz) spectrometer equipped with an Aspect-2000 computer.

The assignment of certain crucial resonances of nonexchangeable hydrogens was accomplished by straightforward spin decoupling experiments in FT mode. For backbone peptide NH protons, the resonances were assigned by underwater decoupling experiments in the correlation mode (Dadok et al., 1972). The chemical shifts of various resonances were measured with respect to TSP as the internal reference. The observed chemical shifts were corrected for the titration of this reference (DeMarco, 1977) and were finally expressed with respect to the position of the TSP peak under acidic conditions.

Transfer of solvent saturation experiments were performed on the 250-MHz spectrometer in the correlation mode at ambient probe temperature (30 ± 1 °C) (Krishna et al., 1979, 1980a,b). The apparent spin–lattice relaxation times of NH hydrogens of UB5 in H_2O were measured at the same temperature on the Brandeis University 270-MHz spectrometer employing the 2–1–4 pulse sequence (Redfield, 1978; Redfield et al., 1975). Relaxation times have not been corrected for the small difference in resonance frequencies (Krishna et al., 1979, 1980a,b). The apparent relaxation rates of all the resonances to the low field of the water peak were simultaneously measured by a saturation recovery experiment employing a sawtooth frequency sweep generator to presaturate the NH and aromatic CH proton resonances. The absence of perturbation of the water signal by the preirradiation was confirmed. A homospoil pulse, applied τ seconds after presaturation, was followed by the 2–1–4 observation pulse after allowing a short interval for recovery from the spoil pulse. The apparent relaxation rates were estimated by a least-squares fitting of the experimental data points (resonance intensity change vs. τ) on a semilogarithmic scale to a straight line.

Results and Discussion

(*1*) *Resonance Assignments.* Figure 1 shows a typical 270-MHz proton NMR spectrum (0.8–5.1 ppm) of UB5 in D_2O. The various resonances have been assigned on the basis of (1) multiplet structure, (2) pH dependence of chemical shifts, and (3) spin-decoupling experiments.

The doublet at 4.1 ppm must belong to Ile $C^\alpha H$ proton since Ile is the only residue in UB5 with a single β proton. De-

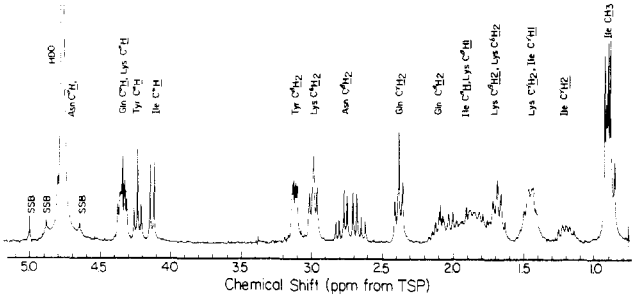

FIGURE 1: The 270-MHz proton FT NMR spectrum (0.8–5.1 ppm) of UB5 (Tyr-Asn-Ile-Gln-Lys) in D_2O, pH 2.5.

coupling of this resonance identified the Ile $C^\beta H$ proton resonance at ~1.9 ppm. The tyrosine $C^\alpha H$ resonance was identified by its $pK_a = 7.42$ which is in the characteristic range for N-terminal α-amino ionization in polypeptides (Greenstein & Winitz, 1961). Decoupling of the Tyr $C^\alpha H$ resonance identified the Tyr $C^\beta H_2$ resonance at 3.1 ppm. The Lys $C^\alpha H$ resonance was identified by its $pK_a = 3.51$, which is in the range expected for C-terminal α-carboxylate group ionization in polypeptides (Greenstein & Winitz, 1961). Tyrosine and asparagine are the only two residues in UB5 whose α- and β-proton resonances are expected to show ABX-type patterns (Pople et al., 1959). Thus, the multiplet structures at 3.1 and 2.7 ppm, each of which corresponds to the AB part of an ABX spectrum, belong to the β protons of these two residues. Since the multiplet structure at 3.1 ppm has already been assigned to Tyr $C^\beta H_2$, the remaining multiplet at 2.7 ppm must belong to Asn $C^\beta H_2$ hydrogens. The Asn $C^\alpha H$ resonance was identified as buried under the solvent peak, since blind decoupling of the solvent peak produced a collapse of the Asn $C^\beta H_2$ multiplet to an AB quartet. The remaining $C^\alpha H$ peak at 4.36 ppm was identified by exclusion as belonging to glutamine. Decoupling of Gln $C^\alpha H$ identified its $C^\beta H_2$ resonance position at 2.1 ppm, which, in turn, helped identify the Gln $C^\gamma H_2$ resonance at 2.37 ppm through decoupling. The triplet at 2.98 ppm has been assigned to Lys $C^\epsilon H_2$. This assignment is consistent with that in the chemical shift data compilation of McDonald & Phillips (1969) and is further confirmed by the high-field shift of this peak for pH >9 due to the titration of the side-chain terminal amino group. It is also consistent with a similar assignment in TP5 for this residue (Krishna et al., 1980a). The resonance group at 0.9 ppm with an intensity of six protons has been assigned to the methyl protons of isoleucine. The remaining resonances in the region between 1.1 and 2.1 ppm have been identified by successive decoupling of the previously assigned resonances. Assignment of resonances in this range is complicated by the severe overlap of multiplets. Difference double resonance experiments (Gibbons et al., 1975) should enable a more precise identification of the resonance multiplets associated with each proton. This procedure, however, does not work too well if the chemical shift difference between the observed and decoupled protons is small enough to result in significant Block–Siegert shifts (Noggle & Schirmer, 1971). Alternately, two-dimensional *J*-resolved spectroscopy (2D-*J*) (Aue et al., 1976; Nagayama et al., 1977; Freeman & Morris, 1979) and two-dimensional spin–echo-correlated spectroscopy (2D-SECSY) methods (Nagayama et al., 1979) may be employed to characterize the NMR spectrum. The 2D-*J* method enables a precise determination of chemical shifts and coupling constants, while the 2D-SECSY technique permits one to trace the *J* connectivities between various multiplets. Figure 2 shows the 2D-*J* spectrum of UB5/D_2O obtained at 400 MHz. The projection of the

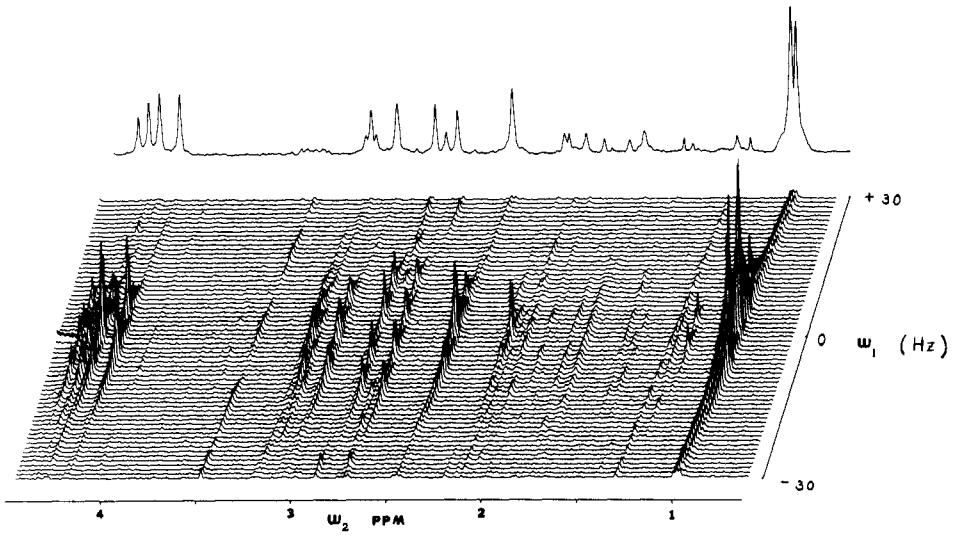

FIGURE 2: Two-dimensional *J*-resolved NMR spectrum of UB5/D_2O (pH 2.9). The spectrum shows the magnitudes and was calculated from 128 × 8192 data points. The ω_2 axis, corresponding to chemical shifts with respect to TSP (0 ppm), is also shown. The ω_1 axis shows the *J* splittings. The top spectrum obtained by a projection onto the ω_2 axis corresponds to a decoupled ^1H NMR spectrum. Apparent in this spectrum are additional transitions associated with second-order effects of scalar coupling (see text).

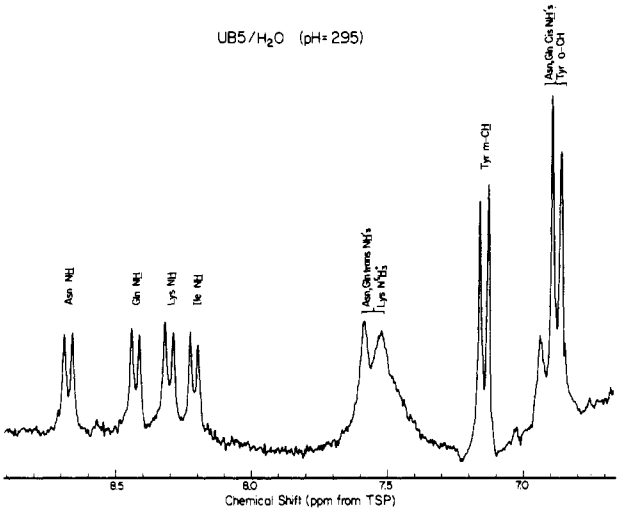

FIGURE 3: The 250-MHz correlation NMR spectrum of UB5 in H_2O showing the low-field proton resonance.

two-dimensional spectrum onto the ω_2 axis corresponding to a completely proton-decoupled spectrum of UB5 is also shown in this figure. The considerable simplification of the spectral region is readily apparent, permitting us an easier determination of the chemical shifts (e.g., the peaks at 2.14 and 2.02 ppm and those at 1.93 and 1.79 ppm are associated with the β protons of Gln[4] and Lys[2], respectively). This method works best for weakly coupled spin systems. For strongly coupled spin systems, the 2D-*J* spectrum can be considerably more complicated due to the appearance of additional transitions (Bodenhausen et al., 1978; Kumar, 1978). Some of these additional transitions are also apparent in the 2D-*J* spectrum of UB5 (e.g., see the β-proton region of asparagine and tyrosine).

The low-field region proton resonance spectrum of UB5 in H_2O is shown in Figure 3. Various backbone NH resonances have been assigned by underwater decoupling of the corresponding $C^{\alpha}H$ resonances in the correlation mode (Dadok et al., 1972). The two doublets at 6.85 and at 7.15 ppm have been assigned to the ortho and meta protons, respectively, of tyrosine (McDonald & Phillips, 1969). The broad resonance at 7.5 ppm has been assigned to the side-chain $N^{\zeta}H_3^+$ protons of lysine. The relatively sharp resonance at 7.6 ppm has been

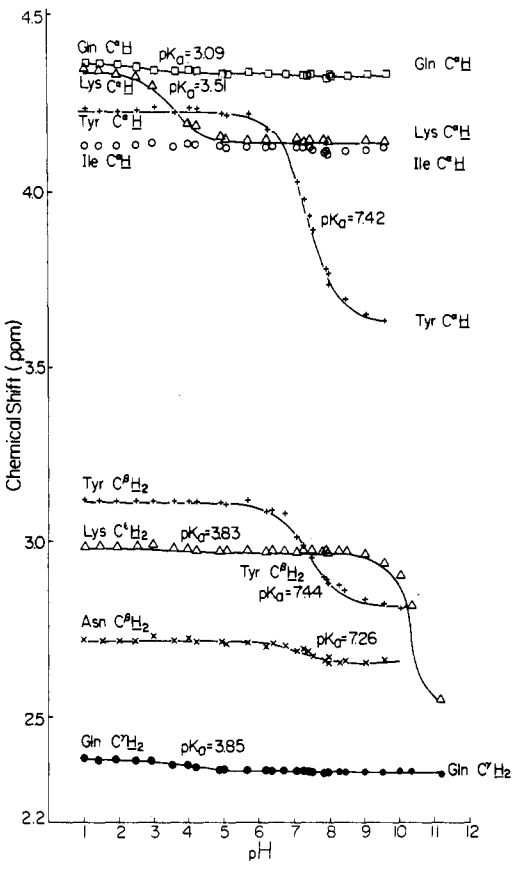

FIGURE 4: pH titration shifts of high-field proton resonances of UB5 in D_2O.

assigned to the trans NH protons of the primary amides in asparagine and glutamine residues. Resonances for the two cis primary amide protons appear at ~6.93 and at ~6.85 ppm (under Tyr *o*-CH resonance).

(2) pH Titration Data. Figures 4 and 5 show the changes of chemical shifts of various well-resolved resonance peaks as a function of pH. The pK_a values were estimated by least-squares fitting to the standard titration equation. All of the observed titration shifts are associated in a straightforward manner with the inductive effect of neighboring ionization sites. Thus, the pK_a values of 7.42 and 3.51 for N-terminal Tyr $C^{\alpha}H$

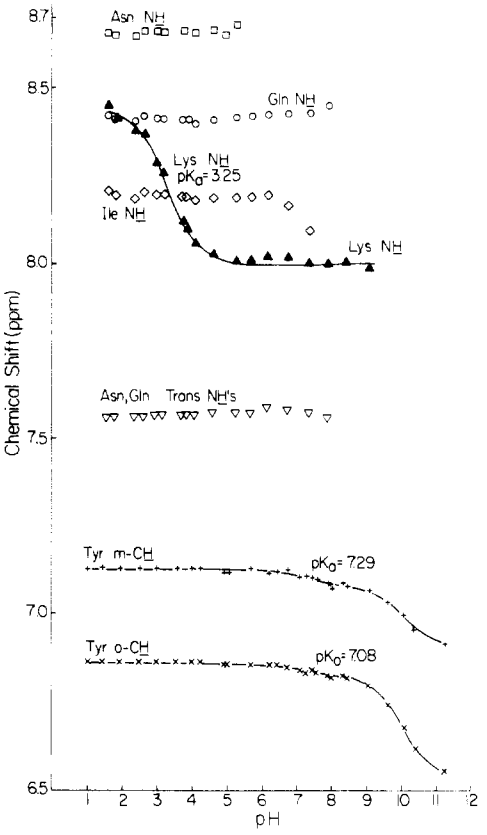

FIGURE 5: pH titration shifts of low-field proton resonances of UB5 in H_2O.

Table I: Vicinal Coupling Constant Data and Possible Sets of Torsional Angles for UB5 Fragment[a]

residue	J_{cor}[b] (Hz)	torsional angles (ϕ) (deg)[c]
Asn[2] NH	8.3 ± 0.4	38–80, −86 to −94, −150 to −160
Ile[3] NH	8.0 ± 0.4	35–83, −84 to −91, −152 to −161
Gln[4] NH	7.7 ± 0.2	34–51, 65–85, −83 to −90, −153 to −162
Lys[5] NH	7.9 ± 0.5	35–83, −84 to −91, −152 to −161

[a] Values of torsional angles were derived by assuming a single conformation and using the Karplus curve given in Figure I-5 of the review article by Bystrov (1976). [b] $J_{cor} = 1.09 J_{obsd}$ where J_{obsd} is the observed doublet separation and 1.09 refers to the correction for substituent effects (Bystrov, 1976). The pH range of 1.5–3.5 was used to measure the coupling constants as the amide proton resonances exhibited broadening for pH >3.5. The NH doublet separations have been corrected to take into account partial overlap of lines in the manner indicated by Bystrov (1976). [c] The additional dispersion arising from the error limits on J values has not been included in the values shown for torsional angles.

and C-terminal Lys $C^{\alpha}H$ resonances, respectively, are in the range expected for polypeptides (Greenstein & Winitz, 1961). Both Tyr $C^{\beta}H_2$ and Asn $C^{\beta}H_2$ resonances experience the titration of N-terminal α-amino group. The tyrosine ortho and meta protons exhibit the titration of both the N-terminal amino group and the phenolic group. Gln $C^{\alpha}H$ and $C^{\gamma}H_2$ resonances show small high-field shifts which may be associated with the ionization of the C-terminal carboxylate group. Lysine $C^{\epsilon}H_2$ experiences the titrations of both C-terminal carboxylate group and the side-chain amino group next to it.

(*3*) *Vicinal Coupling Constants.* The various NH–$C^{\alpha}H$ coupling constants measured for UB5 fragment are listed in Table I. The magnitudes of the coupling constants are compatible with that of conformationally averaged values. Various possible backbone torsional angles (ϕ) that may be realized if a single conformation is assumed are also listed in Table I. We have also measured the vicinal $C^{\alpha}H$–$C^{\beta}H_2$ coupling

Table II: Vicinal Coupling Constants and Derived Populations of Rotamers about the C^{α}–C^{β} Bond of Tyr and Asn of UB5 at 277 K

	calcd $^3J^{HH}$ (Hz)			populations[a]		
residue	J_{AX}	J_{BX}	J_{AB}	I	II	III
Tyr	6.97	7.19	−14.41	0.40	0.42	0.18
Asn	6.06	8.23	−15.5	0.31	0.51	0.18

[a] Determined by using standard Pachler analysis (Pachler, 1964) and the assumed values (obtained from model compounds) of $J_g^{HH} = 2.56$ Hz and $J_t^{HH} = 13.6$ Hz. The rotamers I–III correspond to torsional angles $\chi = 180$ (I), −60 (II), and 60 (III). Convention: the B proton resonance occurs at higher field.

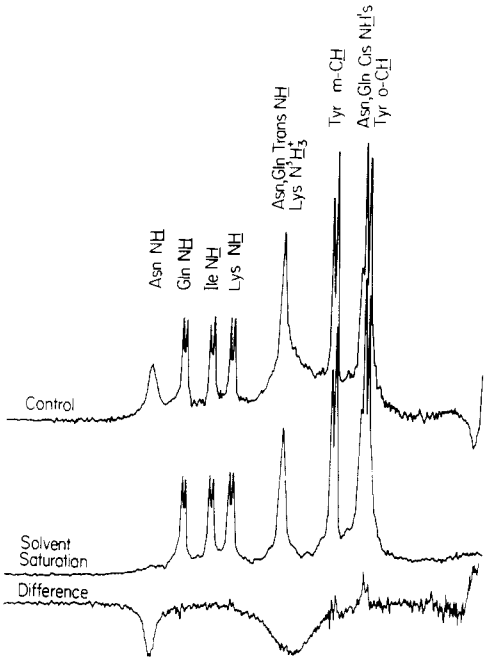

FIGURE 6: Typical transfer of solvent saturation experiment performed in the correlation NMR mode on low-field resonances of UB5 in H_2O. The top trace is the control spectrum (single resonance), the middle trace is the double-resonance spectrum obtained after steady-state saturation of the H_2O resonance, and the bottom trace is the difference spectrum showing the intensity changes; pH 4.89.

constants for the tyrosine and the asparagine residues. From a Pachler-type analysis (Palcher, 1964) of these coupling constants, the various rotamer populations have also been estimated. The coupling constants and the derived rotamer populations are given in Table II. It is clear that two of the three rotamers are significantly populated for each of the residues, with a slight preference for one of the rotamers indicated in the case of Asn. The rotamer population analysis shown here is valid only for classical rotamers. Significant contributions from nonclassical rotamers would invalidate the results.

(*4*) *Amide Hydrogen Exchange Rates.* The backbone amide hydrogen exchange rates of UB5 have been measured by transfer of solvent saturation and saturation recovery NMR experiments (Waelder et al., 1975; Krishna et al., 1979, 1980a–c). Figure 6 shows a typical transfer of solvent saturation experiment on UB5. In the difference spectrum (bottom trace), the negative peaks correspond to a transfer of saturation from the solvent protons to Asn NH and Lys $N^{\zeta}H_3^+$ protons which exhibit significant chemical exchange at the pH value chosen. Tyrosine ortho and meta protons show positive nuclear Overhauser effects (NOE) due to intermolecular dipole–dipole interactions between these protons and the solvent protons (Krishna & Gordon, 1973a,b; Pitner et al., 1975). A typical

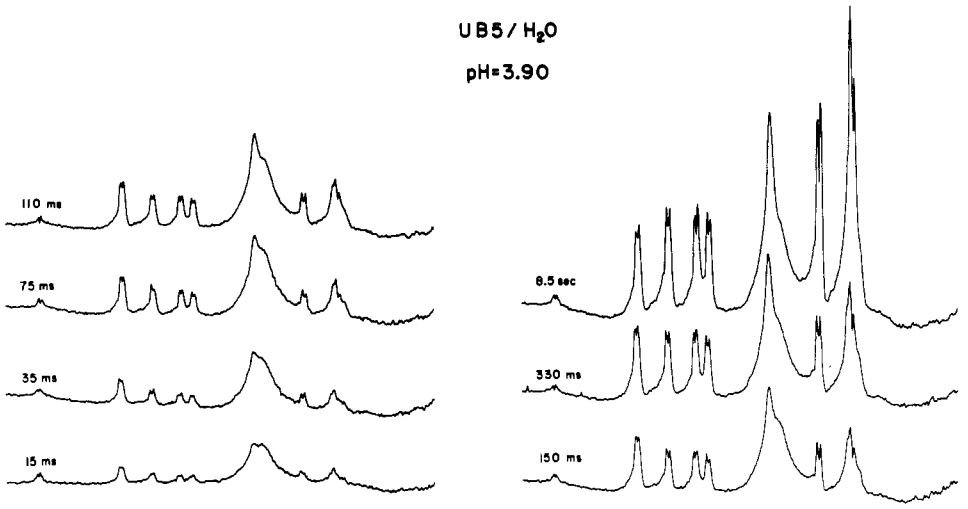

FIGURE 7: Typical saturation recovery experiment performed in the 2–1–4 pulse mode showing recovery of various resonances to their thermal equilibrium values after turning off the saturating radio frequency field. The numbers refer to the delay times.

Table III: Amide Hydrogen Apparent Relaxation Rates (s^{-1}) of UB5 in H_2O at 30 °C

pH	Asn[2] NH	Ile[3] NH	Gln[4] NH	Lys[5] NH
2.10	2.0	2.6	*a*	*a*
3.02	3.0	3.6	3.3	2.5
3.90	5.5	2.8	2.9	2.7
4.90	*b*	3.5	2.7	2.7
6.17	*b*	6.0	6.9	2.4
6.71	*b*	8.5	10.3	2.9

[a] Individual relaxation rates could not be estimated due to resonance overlap. [b] Recovery is too fast to be measurable with the saturation recovery method.

saturation recovery experiment on the low-field resonances of UB5 is shown in Figure 7. The saturation recovery for each amide hydrogen has been found to be described by a single exponential function, within experimental error. The dynamical behavior of most linear oligopeptides appears to be consistent with the assumption of a high-motility limit of conformational transitions (Krishna et al., 1980b,c). In this limit, we have shown earlier (Krishna et al., 1979, 1980b,c) that the fractional intensity change (η) in the intensity of the amide hydrogen magnetization, M_z, under steady-state saturation of the solvent is

$$\eta = \frac{M_z - M_0}{M_0} = -\frac{\langle K \rangle}{\langle R \rangle + \langle K \rangle} \quad (1)$$

The recovery of the amide hydrogen magnetization in a selective saturation recovery experiment is

$$M_z(t) = M_0[1 - e^{-(\langle R \rangle + \langle K \rangle)t}] \quad (2)$$

In the above equations $\langle R \rangle$ and $\langle K \rangle$ are respectively the conformationally averaged values of the spin–lattice relaxation rate and the exchange rate. They are defined by

$$\langle R \rangle = \sum_i P_i R_i$$

$$\langle K \rangle = \sum_i P_i K_{iS} = K_{ex} \quad (3)$$

where P_i is the fractional population of the ith conformer and R_i and K_{iS} are the spin–lattice relaxation rate and chemical exchange rate, respectively, of the amide proton in the ith conformation. The summation in eq 3 runs over all the conformations. The exponent in eq 2 is the apparent relaxation rate, and the experimentally measured values of these for the four amide protons of UB5 are listed in Table III. The

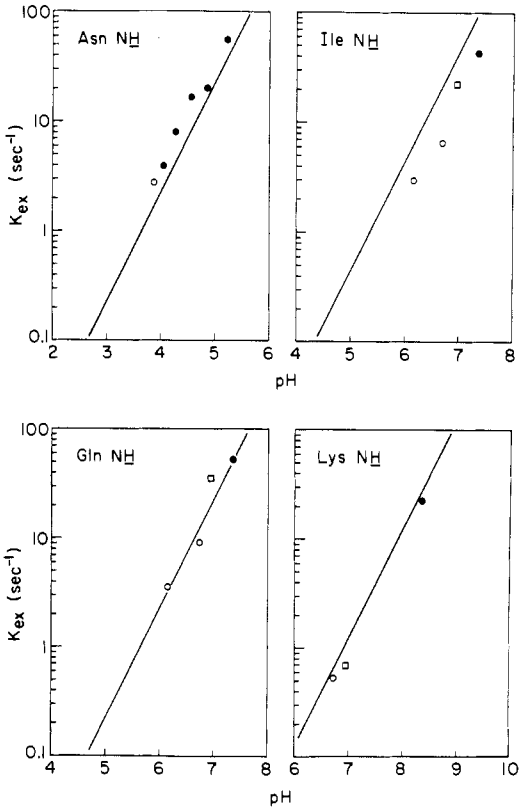

FIGURE 8: Comparison of observed amide hydrogen exchange rates of UB5 with predicted rates (solid lines) for solvated peptides at 30 °C. The predicted rates were calculated in the manner indicated elsewhere (Krishna et al., 1980b). Open circles (○) refer to transfer of solvent saturation data. Solid circles (●) refer to rates estimated from the line widths. Additional measurements at 400 MHz using the transfer of solvent saturation method are indicated by squares (□).

single-exponential nature of the recovery curves and the observed pH dependence of the apparent relaxation rates are compatible with the assumption of a high-motility limit (Hvidt, 1964; Hvidt & Neilsen, 1966) of conformational transitions for the UB5 fragment. The exchange rates K_{ex} ($=\langle K \rangle$) of the individual amide hydrogens determined from the experiments by using eq 1 and 2 are given in Table IV.

The predicted exchange rates of UB5 for the fully solvated peptide have been calculated on the basis of the model compound data of Molday et al. (1972). Figure 8 shows a com-

Table IV: Amide Hydrogen Exchange Rates (K_{ex}) of UB5 in H_2O at 30 °C

pH	rates (s^{-1})[a] Asn[2] NH	Ile[3] NH	Gln[4] NH	Lys[5] NH
3.90	2.8	0	0	0
4.04	3.9[b]	0	0	0
4.29	8.0[b]	0	0	0
4.57	16.7[b]	0	0	0
4.90	20.0[b]	0	0	0
5.22	55.6[b]	0	0	0
6.17		3.0	3.5	0
6.71		6.6	9.1	0.5
6.94[c]		22.8	35.3	0.7
7.34		46.2[b]	52.5[b]	
8.36				22.3[b]

[a] A zero rate implies that the amide proton exchange rate is negligible compared to its spin-lattice relaxation rate. [b] Estimated from the line width as $K_{ex} \simeq \pi(\Delta_{ex} - \Delta_{nex})$ where Δ_{ex} and Δ_{nex} are the full widths (in hertz) at half-height of the amide hydrogen in the presence and absence (i.e., negligible) of chemical exchange, respectively. [c] Measurements performed at 400 MHz on a WH-400 NMR spectrometer.

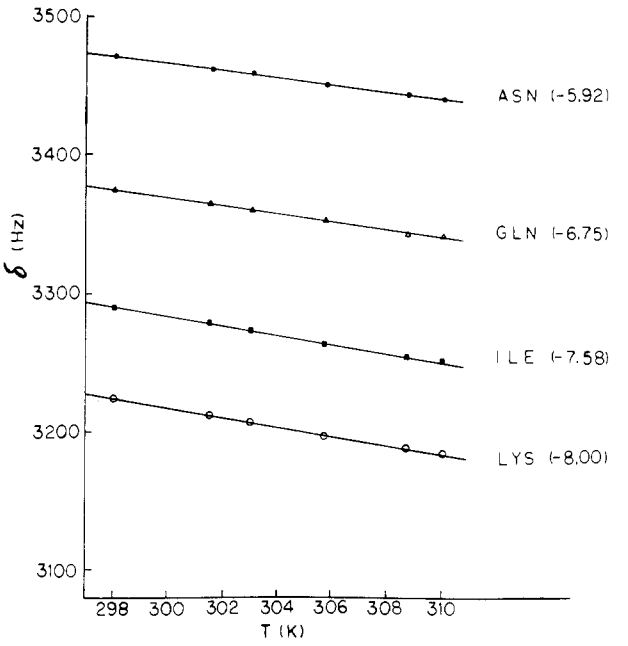

FIGURE 9: Temperature dependence of amide hydrogen chemical shifts (δ) for UB5. The numbers in parentheses refer to the slopes (ppb/K).

parison of the experimental data points with the predicted curves. It is observed that all the four backbone amide hydrogens, Asn NH, Ile NH, Gln NH, and Lys NH, do not exhibit any significant acid catalysis in the pH range 3–8. This observation is consistent with the known behavior of peptides which have minimum exchange rates normally between pH 2 and 3, and consequently only base catalysis is observed for peptide protons at pH >3.5. The experimental exchange rates for all these four amide protons are all in reasonable agreement (within a factor of 2) with the rates calculated from the model compound data. The rate constant for Asn NH is largest of all, due mainly to inductive effect resulting from the positively charged N-terminal α-ammonium group. The predicted second-order base-catalyzed rate constant, k_{OH}, for Asn NH is 1.6×10^{10} M^{-1} s^{-1}, and such a rate constant is characteristic of diffusion-limited exchange process (Molday & Kallen, 1972). For Lys NH, the hydrogen-exchange rates are very small compared to other amide hydrogens. The inductive effect of the proximal negatively charged α-carboxylate group is expected to decrease the base-catalyzed exchange rate of the adjacent peptide group (Molday et al., 1972).

(5) *Temperature Dependence of Amide Hydrogen Chemical Shifts.* The temperature dependence of amide hydrogen chemical shifts has been used in the past as an indicator of solvent accessibility of these hydrogens (Kopple et al., 1969; Urry & Ohnishi, 1969; Llinas et al., 1970; Urry et al., 1975). Amide protons that were internally hydrogen bonded or protected from the solvent were expected to show reduced temperature dependence compared to solvated hydrogens. However, inconsistencies between temperature coefficients and chemical exchange rates as criteria of solvent exposure have also been noted in literature (Pitner & Urry, 1972; Glickson, 1972; Bleich et al., 1973). With this reservation in mind, we have examined the temperature dependence of the amide hydrogen chemical shifts of UB5 with a view to detect any gross differences in their behavior. The results are shown in Figure 9. All the secondary amide NH protons shift to the high field on increasing the temperature, with a small gradation in the temperature coefficients from the high-field Lys NH resonance which shifts fastest to the low-field Asn NH resonance which shifts slowest. There are no gross differences in the behavior of these amide hydrogens [unlike, for example, those observed in gramicidin S (Urry et al., 1975)], and we thus conclude that the temperature-dependence measurements did not provide any evidence contrary to the conclusion arrived at from the exchange data.

Conclusions

The magnitudes of the various NH–C$^{\alpha}$H vicinal coupling constants, the single-exponential behavior of the amide hydrogen saturation recovery curves, and the observed pH dependence of the exchange rates are all compatible with the assumption of a highly motile conformational equilibrium for UB5 in aqueous solution. The exchange rate measurements suggest that all the four amide hydrogens (Asn NH, Ile NH, Gln NH, and Lys NH) remain essentially in a solvated environment within this conformational equilibrium.[2] The temperature dependence of the amide hydrogen chemical shifts did not provide any additional information. The side chains of Asn and Tyr appear to populate two rotamers predominantly, with a slight preference for one of the rotamers indicated in the case of Asn. Further investigations, especially involving intramolecular nuclear Overhauser effect experiments (Noggle & Schirmer, 1971; Krishna et al., 1978; Huang et al., 1981), ^{13}C relaxation time measurements, and conformational energy calculations are in progress to gain more quantitative information on the solution dynamics of UB5. Ultimately, studies of various agonist and antagonist analogues of UB5 and their conformationally constrained derivatives are required to correlate its solution conformation to the biological activity. It is interesting to note that the solution conformation of UB5 appears to differ from that of another cell-differentiating peptide fragment, TP5 (Arg-Lys-Asp-Val-Tyr), the

[2] We would also like to note that the backbone vicinal coupling constants as well as the secondary amide hydrogen exchange rates could be rationalized by the assumption of a single preferred conformation for UB5 in which the amide hydrogens are solvated and the torsional angles (φ) are compatible with the measured $^3J_{NC^{\alpha}}$ values. However, for small linear peptides like UB5, such a situation is unlikely to occur unless there exist strong intramolecular interactions that stabilize the conformation. By showing that the backbone amide hydrogens are solvated, we have eliminated the possibility of conformational stability through hydrogen bonds involving these backbone amide protons. Further studies involving primary amide hydrogen exchange rates, ^{13}C relaxation time, and nuclear Overhauser effect experiments should help to determine the nature of any other interactions that might stabilize the conformation.

conformation of which is stabilized by two intramolecular hydrogen bonds in aqueous solutions (Krishna et al., 1980a).[3] TP5 is the active fragment of thymopoietin, the thymic hormone that induces selective differentiation of T lymphocytes (Basch & Goldstein, 1974). Thymopoietin is believed to bind to a subset of acetylcholine receptors (Goldstein, 1974) whereas ubiquitin binds to β-adrenergic receptors.

References

Aue, W. D., Karhau, J., & Ernst, R. R. (1976) *J. Chem. Phys. 64*, 4226.

Basch, R. S., & Goldstein, G. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1474.

Bleich, H. E., Galardy, R. E., Printz, M. P., & Craig, L. C. (1973) *Biochemistry 12*, 4950.

Bodenhausen, G., Freeman, R., Morris, G. A., & Turner, D. L. (1978) *J. Magn. Reson. 31*, 75.

Bundi, A., & Wuthrich, K. (1979) *Biopolymers 18*, 285.

Bystrov, V. F. (1976) *Prog. Nucl. Magn. Reson. Spectrosc. 10*, 41.

Cook, W. J., Suddath, F. L., Bugg, C. E., & Goldstein, G. (1979) *J. Mol. Biol. 130*, 353.

Dadok, J., & Sprecher, R. F. (1974) *J. Magn. Reson. 13*, 243.

Dadok, J., Von Dreele, P. H., & Scheraga, H. A. (1972) *J. Chem. Soc., Chem. Commun.*, 1055.

DeMarco, A. (1977) *J. Magn. Reson. 26*, 527.

Freeman, R., & Morris, G. A. (1979) *Bull. Magn. Reson. 1*, 5.

Gershwin, M. E., Kruse, W., & Goldstein, G. (1979) *J. Rheumatol. 6*, 610.

Gibbons, W. A., Crepeaux, D., Delayre, J., Dunand, J., Hajdukovic, G., & Wyssbrod, H. R. (1975) in *Peptides: Chemistry, Structure and Biology* (Walter, R., & Meienhofer, J., Eds.) p 127, Ann Arbor Science, Ann Arbor, MI.

Glickson, J. D. (1972) in *Peptides: Chemistry and Biology* (Meienhofer, J., Ed.) p 124, Ann Arbor Science, Ann Arbor, MI.

Goldknopf, I. L., & Busch, H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 864.

Goldstein, G. (1974) *Nature (London) 247*, 11.

Goldstein, G., Scheid, M., Hammerling, U., Boyse, E. A., Schlesinger, D. H., & Niall, H. D. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 11.

Greenstein, J. P., & Winitz, M. (1961) *Chemistry of the Amino Acids*, Wiley, New York.

Gupta, R. K., Ferretti, J. A., & Becker, E. D. (1974) *J. Magn. Reson. 13*, 275.

Hamilton, J. W., & Rouse, J. B. (1980) *Biochem. Biophys. Res. Commun. 96*, 114.

Hershko, A., Ciechanorer, A., & Rose, I. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3107.

Huang, D. H., Walter, R., Glickson, J. D., & Krishna, N. R. (1981) *Proc. Natl. Acad. Sci. U.S.A.* (in press).

Hunt, L. T., & Dayhoff, M. O. (1977) *Biochem. Biophys. Res. Commun. 74*, 650.

Hvidt, A. (1964) *C. R. Trav. Lab. Carlsberg 34*, 299.

Hvidt, A., & Nielsen, S. O. (1966) *Adv. Protein Chem. 21*, 287.

Jenson, J., Goldstein, G., & Breslow, E. (1980) *Biochim. Biophys. Acta 624*, 378.

Kagan, W. A., Siegal, F. P., Gupta, S., Goldstein, G., & Good, R. A. (1979) *J. Immunol. 122*, 686.

Kopple, K. D., Ohnishi, M., & Go, A. (1969) *Biochemistry 8*, 4087.

Krishna, N. R., & Gordon, S. L. (1973a) *J. Chem. Phys. 58*, 5687.

Krishna, N. R., & Gordon, S. L. (1973b) *J. Chem. Phys. 59*, 4569.

Krishna, N. R., Agresti, D. G., Glickson, J. D., & Walter, R. (1978) *Biophys. J. 24*, 791.

Krishna, N. R., Huang, D. H., Glickson, J. D., Rowan, R., & Walter, R. (1979) *Biophys. J. 26*, 345.

Krishna, N. R., Huang, D. H., Chen, D. M., & Goldstein, G. (1980a) *Biochemistry 19*, 5557.

Krishna, N. R., Huang, D. H., & Goldstein, G. (1980b) *Appl. Spectrosc. 34*, 460.

Krishna, N. R., Goldstein, G., & Glickson, J. D. (1980c) *Biopolymers 19*, 2003.

Kuehl, L., Lyness, T., Dixon, G. H., & Levy, W. B. (1980) *J. Biol. Chem. 255*, 1090.

Kumar, A. (1978) *J. Magn. Reson. 30*, 227.

Lenkinski, R. E., Chen, D. M., Glickson, J. D., & Goldstein, G. (1977) *Biochim. Biophys. Acta 494*, 126.

Llinas, M., Klein, M., & Neilands, J. B. (1970) *J. Mol. Biol. 52*, 399.

Matsui, S. I., Seon, B. K., & Sandberg, A. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6369.

McDonald, C. C., & Phillips, W. D. (1969) *J. Am. Chem. Soc. 91*, 1513.

Molday, R. S., & Kallen, R. G. (1972) *J. Am. Chem. Soc. 94*, 6739.

Molday, R. S., Englander, S. W., & Kallen, R. G. (1972) *Biochemistry 11*, 150.

Nagayama, K., Wuthrich, K., Bachmann, P., & Ernst, R. R. (1977) *Biochem. Biophys. Res. Commun. 78*, 99.

Nagayama, K., Wuthrich, K., & Ernst, R. R. (1979) *Biochem. Biophys. Res. Commun. 90*, 305.

Noggle, J. H., & Schirmer, R. E. (1971) *The Nuclear Overhauser Effect: Chemical Applications*, Academic Press, New York.

Pachler, K. G. R. (1964) *Spectrochim. Acta 20*, 581.

Pitner, T. P., & Urry, D. W. (1972) *Biochemistry 11*, 4132.

Pitner, T. P., Glickson, J. D., Rowan, R., Dadok, J., & Bothner-By, A. A. (1975) in *Peptides: Chemistry, Structure and Biology* (Walter, R., & Meienhofer, J., Eds.) pp 159–164, Ann Arbor Science, Ann Arbor, MI.

Pople, J. A., Schneider, W. G., & Bernstein, H. J. (1959) *High-resolution Nuclear Magnetic Resonance*, McGraw-Hill, New York.

Redfield, A. G. (1978) *Methods Enzymol. 49*, 253.

Redfield, A. G., Kunz, S. D., & Ralph, E. K. (1975) *J. Magn. Reson. 19*, 114.

Scheid, M. P., Goldstein, G., & Boyse, E. A. (1978) *J. Exp. Med. 147*, 1727.

Scherrer, H., Seidah, N. G., Benjannet, S., Crine, P., Lis, M., & Chretien, M. (1978) *Biochem. Biophys. Res. Commun. 84*, 874.

Schlesinger, D. H., & Goldstein, G. (1975) *Nature (London) 255*, 423.

Schlesinger, D. H., Goldstein, G., & Niall, H. D. (1975) *Biochemistry 14*, 2214.

Schlesinger, D. H., Goldstein, G., Scheid, M. P., & Bitensky, M. (1978) *Experientia 34*, 703.

Seidah, N. G., Crine, P., Benjannet, S., Scherrer, H., & Chretien, M. (1978) *Biochem. Biophys. Res. Commun. 80*, 600.

[3] We take this opportunity to correct a printer's error on p 5562, second column, second line of Krishna et al. (1980a). The first torsional angle should read $\phi_{i+1} \simeq \mp 80$.

Urry, D. W., & Ohnishi, M. (1969) *Biochem. Biophys. Res. Commun. 36*, 194.
Urry, D. W., Long, M. M., Mitchell, L. W., & Okamoto, K. (1975) in *Peptides: Chemistry, Structure and Biology* (Walter, R., & Meienhofer, J., Eds.) p 113, Ann Arbor Science, Ann Arbor, MI.
Waelder, S. F., Lee, L., & Redfield, A. G. (1975) *J. Am. Chem. Soc. 97*, 2927.
Watson, D. C., Levy, W. G., & Dixon, G. H. (1978) *Nature (London) 276*, 196.
Wilkinson, K. D., Urban, M. K., & Haas, A. L. (1980) *J. Biol. Chem. 255*, 7529.

Modulation by Divalent Metal Ions of the Autocatalytic Reactivity of Adenosinetriphosphatase from Chloroplasts†

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ABSTRACT: A nonlinear, pre-steady-state initial rate of ATP hydrolysis is obtained on the addition of a divalent metal ion–ATP complex to a heat-activated coupling factor 1 isolated from chloroplasts. The acceleration of the initial rate follows first-order kinetics. The observed first-order kinetic constant (k_{obsd}) changes with the concentration of the substrate, reaching half-maximal value at the K_m for ATP hydrolysis. Preincubation of the enzyme with divalent metal ions decreases the k_{obsd} from 1 to 0.04 s^{-1}. Saturation of the divalent metal ion effect was obtained at the micromolar range. It is suggested that the autocatalysis is a result of early stages in ATP hydrolysis which induce conformational changes in the enzyme. Binding of divalent metal ions in the absence of ATP slows down this change.

The CF_1[1] portion of the proton-translocating, reversible ATPase which catalyzes ATP synthesis in chloroplasts consists of five different subunits (Nelson, 1976). This membrane-bound enzyme is highly regulated, as seen from the fact that it does not catalyze ATP hydrolysis unless activated by an electrochemical potential of protons across the membrane in the presence of sulfhydryl reagents (Petrack et al., 1965). The state of activation is quenched by ADP and stabilized by phosphate (Carmeli & Lifshitz, 1972). The acquired reversibility is probably due to conformational changes induced by energization (Ryrie & Jagendorf, 1971). The effect of adenine nucleotides might also be conveyed through changes in conformation since they also increase the capacity of CF_1 to block proton leakage through the ATPase (McCarty et al., 1972).

The isolated enzyme is activated either by heat in the presence of sulfhydryl reagents (Farron & Racker, 1970) or by a mild tryptic digestion (Vambutas & Racker, 1965). Trypsin is suggested to cleave the sensitive ϵ subunit which was shown to act as ATPase inhibitor (Nelson, 1976). ADP acts as an allosteric inhibitor for ATPase activity (Nelson et al., 1972) while several other nucleotides prevent inactivation of the enzyme by cold (McCarty & Racker, 1966) and heat (Farron & Racker, 1970). It is possible that these regulative effects are due to conformational changes which affect the interaction among the various subunits in relation to the ϵ subunit. Subunit interactions are demonstrated by the fact that binding of subunit specific antibody (Nelson et al., 1973) and chemical modification in a single subunit (Deter et al., 1975; McCarty et al., 1972) caused conformational change in the enzyme. Slow changes in the K_i of Co^{3+}–phenanthroline–ATP complex (Hochman et al., 1979) and fluorescent changes on binding of ADP (Girault & Galmiche, 1977) are also indicators of conformational changes in the protein.

In preliminary reports (Carmeli et al., 1978, 1979), we showed that ATPase activity in isolated CF_1 goes through a transient state on addition of substrate. Similar results were obtained in ATPase from yeast mitochondria (Recktenwald & Hess, 1977). The data presented here indicate that the kinetic changes that take place during the early events in ATP hydrolysis could reflect conformational transformations of the enzyme which are expressed as various states of activity. It is suggested that one of these forms of the enzyme is a result of binding of divalent metal ions to the enzyme.

Experimental Procedures

Coupling factor 1 was prepared from lettuce (Romaine) chloroplasts according to the method used for spinach chloroplasts (Lien & Racker, 1971) and stored and prepared for use as indicated earlier (Carmeli et al., 1978). The protein concentration was determined according to the method of Hartree (1972). Following heat activation (Farron & Racker, 1970) the protein catalyzed ATP hydrolysis at a rate of 35 μmol mg^{-1} min^{-1}, indicating the high purity of the enzyme.

ATPase activity was measured spectrophotometrically by using the indicator cresol red for monitoring the acidification of the lightly buffered solution in Aminco DW-2 spectrophotometer equipped with a stopped-flow apparatus as described earlier (Carmeli et al., 1978).

Results

Kinetics of Acceleration of ATPase Activity. The reaction was initiated by mixing the enzyme (plus EDTA) with an equal volume of CaATP containing 0.1 mM free Ca^{2+} and a pH indicator. The decrease in pH quantitatively measures ATPase activity. The earliest ATPase activity is nonlinear with time. As was shown by Schreiber et al. (1979), such curvature can be analyzed if the logarithm of change of ve-

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[1] Abbreviations used: CF_1, coupling factor 1 from chloroplasts (ATPase); Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; AMPPNP, adenyl-5′-yl imidodiphosphate; EDTA, ethylenediaminetetraacetate.

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