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^{13}C Nuclear Magnetic Resonance Study of Molecular Motions and Conformational Transitions in Muscle Calcium Binding Parvalbumins†

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ABSTRACT: ^{13}C nuclear magnetic resonance is used to detect the Ca^{2+} ion controlled conformational transition in muscle calcium binding parvalbumin and to study its intramolecular motions. Nuclear relaxation parameters are used to evaluate the reorientation rates of the protein and some of the amino acid side chains. While peripheral residues exhibit greater motional freedom than the protein interior, an interesting finding is that significant rapid internal motion is present in the phenylalanine rings comprising the hydrophobic core of the protein.

Understanding the function of proteins requires not only knowledge of their structure, but also of the degrees of motional freedom allowed within the structure. In some cases of simple enzymes, knowledge of the static configuration alone may suffice to deduce the catalytic mechanism. However in regulatory and transport proteins, or proteins involved in generating mechanical work, where function is inextricably linked to structural change, knowledge of the available conformational options and segmental mobility is essential.

Many physical, especially spectroscopic methods, can and have been used to detect molecular motions in proteins. Few are suited to describe them in detail. The basic requirement for such a description is that motions of different portions of the molecule be monitored simultaneously. For this reason, high resolution nuclear magnetic resonance is particularly well suited to the study of this problem.

Since the early days of the development of the method, internal motions in polymers have been detected (Bovey, 1959; Jardetzky, 1964a,b) and the particular usefulness of relaxation methods for their investigation had been recognized (Jardetzky and Jardetzky, 1962; Jardetzky, 1964b). In recent years several groups have embarked on a more detailed study of intramolecular motions in proteins (Roberts, 1975). ^1H NMR[1] studies of protein mobility have relied on qualitative features of averaging spectra to place lower limits on the effective rates of internal reorientation (Snyder et al., 1975; Campbell et al., 1975; Wüthrich and Wagner, 1975; Cave et al., 1976). Quantitative interpretations based on relaxation data have been carried out for ^{19}F-labeled proteins (Hull and Sykes, 1975) and for proteins studied by ^{13}C NMR in natural abundance (Allerhand et al., 1971a,b; Opella et al., 1974; Oldfield and Allerhand, 1975a,b; Oldfield et al., 1975) or with site enrichment (Browne et al., 1973; Hunkapiller et al., 1973). These studies have made it apparent that to obtain quantitative information on the motion of individual amino acid side chains, and on changes of these motions with changes in protein conformation, it is necessary to use multiple relaxation parameters to reduce the number of physical models which can be compatible with the data.

^{13}C magnetic resonance provides a particularly informative approach to the problem of internal motions. First, it offers the opportunity of measuring two relaxation parameters (the longitudinal relaxation time T_1 and the nuclear Overhauser enhancement, NOE) with a significantly different functional dependence on the correlation times. The transverse relaxation time T_2 is in principle also useful for this purpose, but is experimentally less accessible for the spectra of large molecules, or generally spectra with limited resolution and signal-to-noise ratios and especially for noise-decoupled ^{13}C spectra. Second, theoretical considerations are greatly simplified for the large class of carbons with directly bonded hydrogens since the dominant relaxation mechanism is dipolar. Third, the distance between the dominant dipoles is given by the C–H bond length, which is constant and known, allowing accurate correlation time calculations at least for isotropic motion. The determination of the rates of internal motion, which are generally anisotropic, is a more complex problem, discussed in more detail below.

Muscle calcium binding proteins (MCBP) have been chosen for this study for several reasons. The MCBPs are of relatively low molecular weight (mol wt ~12 000), highly soluble, and acidic (Pechere et al., 1971). Multiple isotypes are usually isolated from a given species (e.g., there are three major isotypes obtained from common carp (*Cyprinus carpio*)). These isotypes are convenient for comparative studies and greatly aid

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[1] Abbreviations used: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; MCBP, muscle calcium binding proteins; TN-C, troponin trimer complex; ALC, myosin alkaline-extractable light chain protein; cAMP, cyclic adenosine 3′,5′-monophosphate; DEAE, diethylaminoethyl; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N′*-tetraacetic acid; Me_4Si, tetramethylsilane.

in the assignment of individual resonance signals. Finally, MCBPs are known to undergo conformational alterations with the removal of protein-bound calcium (Parello and Pechere, 1971; Parello et al., 1974; Donato and Martin, 1974).

The crystal structure of one of the MCBP isotypes from carp (MCBP-3) has been solved and refined to a nominal 1.9-Å resolution (Kretsinger and Nockolds, 1973). The protein has a rigid structure with several well-defined domains. There are six α-helical sections in the MCBP molecule, designated helices A through F, with a calcium ion coordinated in the polypeptide loop between helices C and D and a second calcium ion in the loop between helices E and F. The CD calcium ion is completely surrounded by protein ligands, while the EF calcium has solvent water for one of the contributing ligands. MCBP-3 contains a tightly knit hydrophobic core composed primarily of the phenyl rings of eight phenylalanine residues.

Apart from being convenient models for the study of internal motions, MCBPs are of considerable interest in their own right. Although their specific function remains unknown, biological processes utilizing these proteins appear to be modulated by calcium ion. There is a close homology between the MCBP class and other high-affinity calcium binding proteins, including the troponin calcium binding component of the troponin trimer complex (TN-C), the myosin alkaline-extractable light-chain protein (ALC) (see Tufty and Kretsinger, 1975; Kretsinger and Nelson, 1976) and the cAMP phosphodiesterase activator protein (Teo and Wang, 1973). A complete description of the structural consequences of calcium removal in one of these homologous proteins could, therefore, lead to a better understanding of the mode of action of an entire class of high-affinity calcium binding proteins.

Methods

Muscular parvalbumins were isolated from the white muscle of common mirror carp (*Cyprinus carpio*), according to the method of Pechere et al. (1971). The three major isotypes, MCBP-2, MCBP-3, and MCBP-5, were separated by chromatography on DEAE-52-cellulose ion-exchange resin, employing a 0.015 M HCl–piperazine buffer (pH 5.7), with a 0.0–0.1 M NaCl linear gradient. The three major parvalbumins were resolved and identified by polyacrylamide slab gel electrophoresis. The identification of MCBP-2, the only major parvalbumin containing a tyrosine residue (Tyr-2), was confirmed by the characteristic ultraviolet absorption spectra at 280 nm (for MCBP-3, $A_{259}/A_{280} = 4.82$ and for MCBP-2, $A_{259}/A_{280} = 1.62$). Parvalbumin isotypes were also identified by amino acid analysis.

Calcium content of the purified parvalbumins, as determined by atomic absorption spectroscopy, indicated the full complement of calcium (2 Ca^{2+} ions/MCBP molecule) (Opella et al., 1974). For experiments on parvalbumin proteins with one-half the native complement of calcium (i.e., calcium coordinated to the CD metal ion binding site but not to the solvent exposed EF site), the calcium was removed with the addition of ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA). In a recent atomic absorption study Nelson et al. (1976) demonstrated that at neutral pH a 20-fold molar excess of EGTA to parvalbumin removes exactly one of the two bound calcium ions from the protein.

All nuclear magnetic resonance experiments were performed at 25.16 MHz on a Varian XL-100 spectrometer equipped with a pulse unit and data system from Nicolet Technology Corp. Broad band noise decoupling was typically 12 W modulated over about 2 kHz centered on the ^1H spectrum. Samples were run in 12-mm OD sample tubes with about 1 ml of total sample volume. The usual protein concentration was 15 mM. The probe temperature was maintained at 22 ± 2 °C. All chemical shifts are reported with respect to an external Me_4Si reference. The pH was measured on a Radiometer Model 26 pH meter and reported values are not corrected for the presence of deuterium in the solvent.

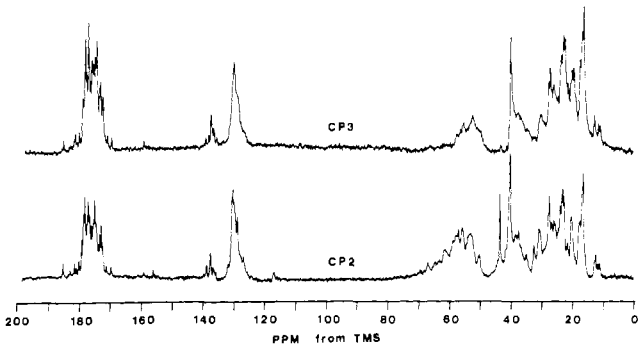

FIGURE 1: Proton decoupled natural abundance carbon-13 nuclear magnetic resonance spectra of MCBP-2 and MCBP-3. All chemical shifts are relative to external Me_4Si. The spectral width is 6.5 kHz and data were collected following 90° rf pulses at 1.245-s intervals. Data points (16K) were collected. The spectra followed from adding 15K of zeroes to the data, multiplication by a negative exponential function corresponding to 2.0-Hz line broadening and Fourier transformation. MCBP-3 (2 calciums/molecule), 20 mM, pH 7.8, 50 000 transients. MCBP-2 (2 calciums/molecule), 15 mM, pH 7.8, 86 000 transients.

Spin–lattice relaxation time (T_1) values were measured by the inversion–recovery technique employing the pulse sequence $(180°-\tau-90°-T)_n$, where τ is the variable delay time between the two applied pulses and T is a time calculated to be at least five times the T_1 being measured (Vold et al., 1968). Nuclear Overhauser enhancement, NOE, values were measured by the gated decoupler technique (Freeman et al., 1972; Opella et al., 1976). The sequence of events in the gated decoupler experiment as used in this work is: The proton decoupler is turned on simultaneously with a 90° rf pulse at the carbon frequency. The free induction decay is collected immediately afterward for the shortest acquisition time consistent with the necessary spectral resolution. Data collection is followed by a delay time during which the decoupler is turned off. This permits the observation of a completely decoupled ^{13}C spectrum without NOE contribution. An account value of the NOE can then be obtained by comparing line intensities with those in a continously decoupled spectrum.

Results and Discussion

Spectral Assignments

The natural abundance carbon-13 NMR spectra of muscle calcium binding parvalbumin, isotypes 2 and 3, are shown in Figure 1. The carboxyl and carbonyl carbon resonances appear between 165 and 190 ppm. A number of these resonances are resolved. All parvalbumins isolated to date contain an invariant arginine residue (Arg-75), the guanido carbon resonance of which appears at 158.8 ppm in these spectra. The resonances from the ten phenylalanine residues constitute the major peak in the aromatic region of the MCBP-3 spectrum (MCBP-3 contains one histidine residue and no tryptophan). The relatively sharp signals from 135 to 140 ppm arise from the γ carbons of the phenylalanines and the heterogeneity of these resonances is indicative of the substantial chemical shift nonequivalence induced by the native protein structure. A number of the phenylalanine γ carbons are also resolved in both isotypes. The phenylalanine ring carbons with bonded hydrogens

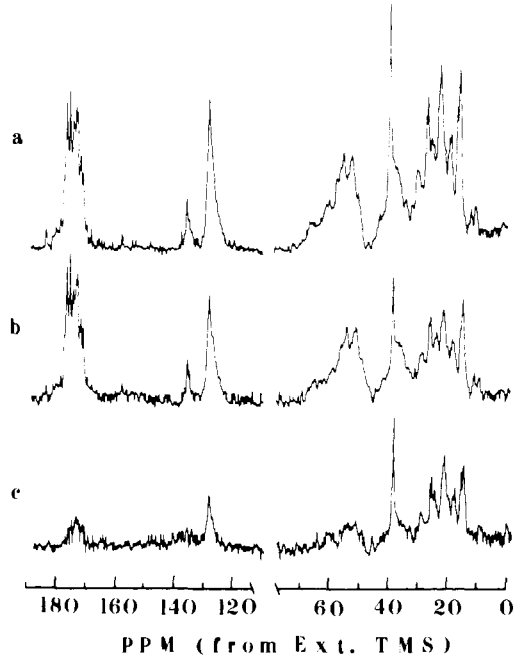

FIGURE 2: Comparison of nuclear Overhauser enhancement (NOE) for different spectral regions of MCBP-3. (a) The spectrum with continuous proton noise irradiation. (b) The gated decoupled spectrum, with an acquisition time of 0.311 s followed by a delay of 2.0 s. It contains no intensity contributions from NOE. (c) The NOE difference (NOED) spectrum obtained by subtracting b from a.

contribute to the broad resonance band centered at 129 ppm. The resonance signals from the α carbons of the protein backbone appear between 50 and 60 ppm. Methylene and methyl carbons occur between 10 and 40 ppm. This upfield region is dominated by the ϵ-methylene carbons of 13 lysine residues (~39 ppm) and by the β-methyl carbons of 20 alanine residues (~16 ppm). The resonances at highest field (10 to 14 ppm) are from the δ-methyl carbons of the five isoleucine residues. Two of these methyl carbons appear as single resonances (10.7 and 11.2 ppm) in both isotypes.

The spectra illustrated in Figure 1 are obtained under conditions of continuous proton decoupling. The observed resonance intensities, therefore, reflect both the number of carbon atoms of a particular type and varying amounts of nuclear Overhauser enhancement (NOE). Such spectra are easily obtained and useful for comparisons of chemical shifts between different MCBP isotypes. However, an accurate analysis of the spectrum requires an evaluation of the NOE contribution by the gated decoupler technique (cf. Methods).

A more detailed assignment of spectral lines is possible by a comparison of the spectra of different isotypes of different amino acid composition, e.g., MCBP-2 and -3. MCBP-2 differs from MCBP-3 at 19 residue locations. These residue changes would not be expected to radically alter the conformation of MCBP-2 relative to MCBP-3, as for the most part, hydrophilic residues replace hydrophilics and hydrophobics replace hydrophobics. The spectra of the two parvalbumin isotypes are, in fact, very similar not only in general appearance, but also with respect to resonance frequencies of signals from individual carbon sites (Figure 1), except where differences in amino acid content appear.

In both MCBP-2 and MCBP-3 a single carboxyl resonance is shifted far downfield (184.6 ppm). This unusually deshielded resonance can be tentatively attributed to the carboxyl carbon of Glu-81 on the basis of two arguments (Opella et al., 1974). Although calcium coordination to carboxyl groups is expected to cause some deshielding of resonances, it is unlikely that any carbon of the eight carboxyl groups involved in coordination has such an unusual relationship to the calcium ion as to result in a shift of this magnitude (see crystal structure data of Kretsinger and Nockolds, 1973). Second, the carboxyl group of Glu-81 is unique because it is involved in an internal ionic bond to the guanido group of Arg-75. Model studies indicate that carboxyl groups involved in intermolecular hydrogen bonding have their resonance shifted downfield in proportion to the basicity of their bonding partner (Maciel, 1965). The only functional groups that are basic enough to account for the observed chemical shift are the amino groups of the 13 lysine residues and the guanido group of the single arginine; however, since all of the lysine amino groups are exposed to the solvent, they are not available for interaction with buried carboxyl groups, leaving Arg-75 as the sole likely candidate to provide a hydrogen bond. The structural homology between MCBP-2 and MCBP-3 is further indicated by the similarity of resonance patterns in the carbonyl resonance region (172 to 178 ppm) since carbonyl chemical shifts are quite sensitive to local conformational environments. Two distinct carbonyl resonances are upfield from the main envelope (170.6 and 169.2 ppm), representing carbons in highly shielded environments compared with the other 106 peptide carbonyl groups. It is known from the crystal structure of MCBP-3 that two carbonyl oxygen atoms, those of Lys-96 and Phe-57, coordinate calcium ions in the EF and CD metal binding loops, respectively. It has also been shown, from small molecule crystal structures, that the coordination of alkali and alkaline earth metals by carbonyl groups decreases the length of the carbon–oxygen bond and increases that of the peptide nitrogen–carbon bond (Van der Helm and Willoughby, 1969). This change in bond length would result in decreased π-bond polarity, increased carbon–oxygen stretching frequency, and increased electronic shielding of the carbonyl oxygen (Maciel, 1965). The attribution of the upfield carbonyl resonances to Phe-57 and Lys-96 is consistent with this information. It is further supported by the finding that upon release of calcium from the EF coordination site the most shielded carbonyl resonance shifts significantly downfield (cf. below).

The aromatic region of the two parvalbumin isotypes is very similar with the exception that MCBP-2 has a tyrosine residue replacing Phe-2 of MCBP-3. The ζ carbon of tyrosine has a chemical shift of 156 ppm at neutral pH. The signal which occurs at 155 ppm in the spectrum of MCBP-2, but not MCBP-3, can be assigned to the ζ carbon of Tyr-2. In addition, the resonances at 120 ppm can be assigned to the two ϵ-ring carbons of Tyr-2. A particularly striking feature of the MCBP-2 spectrum is the apparent resolution of the two ϵ-carbons of Tyr-2. An expansion of the spectral region containing the Tyr-2 ϵ carbons is shown in Figure 2. Although these atoms are chemically equivalent, the two peaks observed in the native protein, with two bound calcium ions, indicate that these carbons exist in different magnetic environments (i.e., the Tyr-2 ring is probably partially buried in the protein interior, with exchange between these environments presumably by rotation of the phenyl ring, being slow on the NMR time scale). Differences in the rates of reorientation of aromatic side chains as revealed by NMR have been noted (Roberts, 1975); it is apparent that Tyr-2 is a clear example of an aromatic group held rigidly within the protein structure when both Ca^{2+}-binding sites are occupied.

The upfield methylene and methyl carbon region is also strikingly similar in the two parvalbumin isotypes. Even the

two upfield shifted δ-methyl carbons of isoleucine (at 10.7 and 11.2 ppm), observed in the MCBP-3 spectrum, are present in the MCBP-2 spectrum as well. Moews and Kretsinger (1975) observed two "short contact" isoleucine methyl groups, the δ-methyl carbon of Ile-58 being 3.56 Å from the δ_1-ring carbon of the Phe-47 and the δ-methyl carbon of Ile-97 being 2.69 Å from the δ-methyl carbon of Leu-86. At the time of the x-ray study it could not be independently shown that there were two unusual isoleucine methyl groups. The observed short distances might have been an artifact of the refinement procedure. A clear result of the present carbon-13 magnetic resonance study is that there are two unusual, probably short contact, isoleucine δ-methyl groups, even though at the present time we cannot distinguish between the two upfield shifted isoleucine methyl groups and assign them individually to Ile-58 and Ile-97.

Determination of Relaxation Parameters and Evaluation of Reorientation Rates

Relaxation theory developed by Bloembergen et al. (1948), Solomon (1955), and Abragam (1961) generally permits the interpretation of measured relaxation parameters (T_1, T_2, NOE) in terms of the time constants of molecular motion. The calculation of these time constants, the correlation times, is relatively straightforward in cases where (1) the dominant relaxation mechanism is known, (2) a single nucleus or a single field is responsible for the relaxation of the observed nucleus, (3) the distance of the relaxed from the relaxing nucleus (in the case of dipolar relaxation) can be accurately determined, (4) a single type of motion is responsible for relaxation, and (5) this motion is isotropic. If any one of these conditions does not hold, interpretation hinges on specific models and approximations and it is not unusual to find more than one model which will account for a given measurement. Relaxation measurements in ^{13}C spectra are particularly well suited for a quantitative analysis of the underlying motions, because (1) at least for carbons with directly bonded hydrogens the first three conditions can be assumed to hold; the dominant relaxation mechanism is dipolar and the largest contribution to the relaxing field is from the bonded hydrogen, and (2) it is feasible to measure at least two relaxation parameters (T_1 and NOE), with a different functional dependence on the correlation time. This makes it possible to ascertain whether the same model and, in particular, the same correlation time can simultaneously account for the two measurements. One can thus reduce the number of plausible models which account for either measurement when taken alone. In the course of this work we have often encountered the case where a highly plausible model and a plausible value of the correlation time would adequately account for the observed values of T_1, but not NOE, or vice versa. Therefore we do not attribute any physical significance to any correlation times that do not account for at least two independently determined relaxation parameters.

It must always be borne in mind that the calculated values of the correlation times will reflect the accuracy of the relaxation measurements. T_1 can usually be determined with sufficient accuracy (5–10%) by the inversion recovery technique (Vold et al., 1968), provided one ensures complete inversion of the observed resonance. On the other hand the customary method for measuring NOE—the comparison of intensities in a decoupled spectrum with those in an undecoupled spectrum—becomes progressively less reliable as the complexity of the spectrum increases. For this reason we have developed a procedure (Opella et al., 1976) based on the gated decoupling experiment of Freeman et al. (1972), which allows the evaluation of the NOE contribution from fully decoupled ^{13}C spectra alone, with an accuracy of 5–10%. As noted under Methods, this technique obtains all spectral data with pulsed proton decoupling to remove ^{13}C–^1H scalar couplings, using prior to data acquisition a delay period of appropriate length with no proton irradiation to ensure ^{13}C resonance intensities without Overhauser enhancement. These intensities are then compared with those measured under conditions of continuous decoupling. The method has a number of advantages over other methods since it is not necessary to add either paramagnetic or diamagnetic impurities (Oldfield et al., 1975) to the protein solution and integration of multiplets is not required. Figure 2 compares (a) the continuously decoupled natural abundance ^{13}C spectrum of MCBP with (b) the gated decoupled spectrum which is identical except for the elimination of NOE. A clear indication of the relative magnitude of NOE for different carbons can be obtained from the difference spectrum (c) where all of the signal intensities are due to the Overhauser effect.

TABLE I: NOE of Different Resonance Regions in MCBP.

Types of Carbon	No. of Carbons	Area % Continuous Decoupling	Area % Gated Decoupling
Carbonyl	133	128 ± 10	100 ± 10
Aromatic	62	172 ± 5	100 ± 14
Aliphatic	315	184 ± 5	105 ± 8

The spectrum without NOE (Figure 2b) can be used to determine the number of carbon atoms in a particular resonance region. Table I summarizes the relative contributions to the integrated intensities due to the various types of carbons and their respective NOE. If the protein behaved like a small molecule in solution, then the area obtained with continous decoupling would be 300% of that measured by gated decoupling for carbons with bonded hydrogens. The smaller observed values of NOE reflect the fact that the motions of the protein are smaller than those of a small molecule. The fact that on more detailed examination (cf. below) different values of NOE are found for different types of carbons consititutes qualitative evidence for internal motion. The measured relaxation parameters mut therefore be analyzed in terms of at least two types of motion: (1) the rotation of the protein as a whole, which can in this case be assumed to be isotropic and (2) internal motions, which are more likely to be anisotropic.

Isotropic Reorientation of MCBP. The rotational diffusion coefficient, D, calculated from the Stokes–Einstein equation adequately describes the reorientation of small globular proteins in solution. In this case the autocorrelation function is a single exponential with a time constant that is the rotational correlation time ($\tau_c = \frac{1}{6}D$). The ^{13}C nuclear relaxation parameters are readily calculated for this type of motion.

The values of T_1 and NOE as a function of isotropic rotational correlation time have been calculated a number of times (Doddrell et al., 1972; Allerhand et al., 1973a,b; Oldfield and Allerhand, 1975a,b; Oldfield et al., 1975; Bauer et al., 1975). In the case of MCBP which has a very high α-helix content it should be possible to obtain an overall reorientation rate of the native protein by considering the α carbons to be sterically restricted so that their effective correlation time is the same as that of the protein. Measurements of the α-carbon relaxation parameters then lead directly to τ_c, the isotropic rotational correlation time, and to the diffusion coefficient measured by other methods.

The experimental T_1 (55 ms) and NOE (1.2) for the α

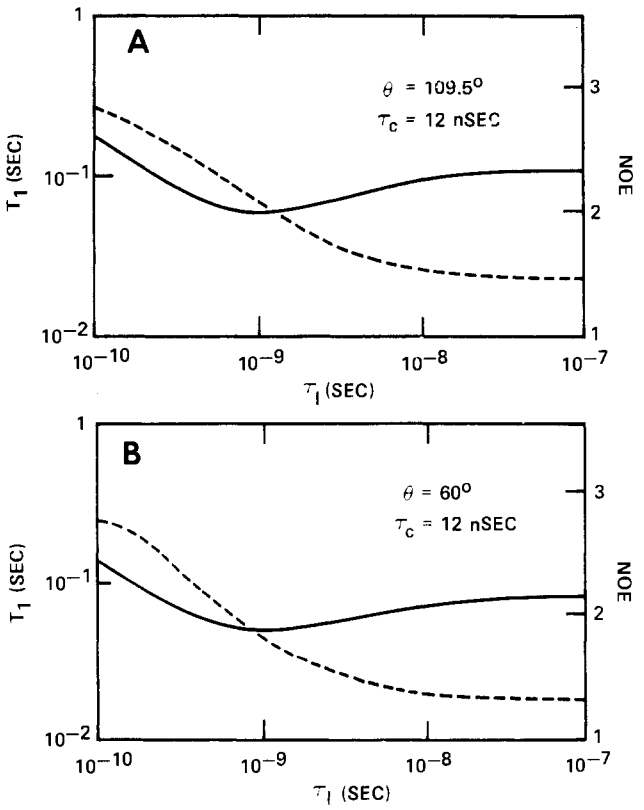

FIGURE 3: Calculated T_1 (solid line) and NOE (dashed line) dependence on the internal reorientation time τ_I for a ^{13}C-H vector inside a rigid protein with an overall isotropic reorientation time τ_c = 12 ns. (A) For rotation about an axis at an angle of 109.5°. (B) For rotation of an angle of 60°.

carbons of MCBP are consistent only with a rotational correlation time of about 12 ns. This value can be compared with the value of 10–12 ns obtained from depolarized light scattering (Bauer et al., 1975), indicating that the two methods are sensitive to the same motion, with the measured correlation times being the same within experimental error. The finding that the correlation time for the α carbons of this protein is the same as that calculated from the overall reorientation supports the assumption that the α carbons are rigidly held in the protein structure. On the basis of the fit of the NMR parameters to the calculated isotropic values and the single Lorentzian line shape for the depolarized Rayleigh spectrum, the tumbling of MCBP can be accurately described as isotropic.

Anisotropic Reorientation: Quantitative Treatment of Intramolecular Motions of Amino Acid Side Chains. The determination of the rates of internal motions of amino acid side chains in a protein structure is a significantly more difficult problem than the calculation of an overall isotropic correlation time. One simple approach to the analysis of the motions of side chain groups is to consider each group as attached to a rotating rigid body. It is possible to calculate the relaxation parameters for one degree of internal rotation superimposed on the overall isotropic reorientation. However, in this case the spectral densities are functions of *all* the components of the rotational diffusion tensor. Following Woessner (1962) the motion of C–H groups can be treated as a diffsional reorientation about an axis attached to a framework which itself tumbles isotropically. The internal rotation is specified by a correlation time, τ_I, and an angle, θ, which the CH vector forms with the axis of internal rotation. In this model, the motions are considered as stochastic diffusion among a large number of equilibrium positions so that the diffusion constant for the internal rotation is the inverse of $6\tau_I$. As for the case of isotropic motion, direct calculation of the relaxation parameters is possible through evaluation of the spectral densities appropriate for the rotation model.

It is clearly necessary to use an accurate value of the overall correlation time in these calculations since the calculated value of τ_I will vary with the assumed value of the isotropic τ_c. In this case the value of 12 ns derived from NMR measurements on α carbons and depolarized light scattering data is used to calculate τ_I. Plots of NOE and T_1 as a function of rate of internal reorientation are given in Figure 3. Two different angles of rotation of ^{13}C–H bond vectors are shown: for tetrahedral carbons (θ = 109.5°) and for aromatic side chains which rotate about the C_β–C_α bond (θ = 60°).

If the T_1 and NOE data for a given side chain are not accounted for by the isotropic τ_c and if a single value of τ_I can be found from the plots in Figure 3 to account for the measurements, it is reasonable to assume that the model applies. However, since most carbons in amino acid side chains can have several different intramolecular motions, the analysis in terms of one degree of internal rotation is restricted to a few structural groups. For example, the β carbons of amino acids would be expected, in general, to fall into the category of single internal rotation with respect to an adjacent α carbon undergoing isotropic reorientation. The β carbons of lysine and valine contribute to the resonance band between 24 and 27 ppm. These signals have a relatively homogeneous NOE of 1.4 and an observed T_1 of 50 ms ($2T_1$ = 100 ms). There are no isotropic correlation times that simultaneously satisfy these two relaxation parameters. This finding by itself is strong evidence for the motions of these carbons being anisotropic in nature. If the β-carbon–hydrogen dipolar vector rotates about the C_β–C_α bond, then the angle of rotation (θ) can be reasonably defined as 109.5°, the value for a tetrahedral carbon. Using the functional dependence of T_1 and NOE on the internal correlation time shown in Figure 3, the experimental values can be accounted for with an internal rotation of τ_I = 10 ns. A similar treatment applied to the resonances arising from the carbons of Asp, Leu, and Phe results in the finding of a considerably slower rate of internal reorientation of C_β–C_α bonds (10–20 ns).

The β carbons of alanine residues would also be expected to have internal motions that could be reasonably explained by a single internal rotation superimposed on the overall motions of the protein. However, NT_1 = 650 ms and NOE = 1.8 for MCBP-3, and this cannot be accounted for with either the isotropic model or the single internal rotation model. There are several possible sources for this failure of the simple model of the motion. The alanine resonance peak at about 16 ppm results from 20 different reorientation rates about the same axis, even though the chemical shift is relatively homogeneous. It is also possible that the motions of the methyl groups are not diffusional in nature, or that the appropriate angle for internal rotation is not 109.5°.

Most side chain carbon relaxation parameters do not fit at all either to the isotropic diffusion or the single internal rotation model. If the carbons are not in the β position (i.e., all carbons further removed from the protein main chain), it is inappropriate to use a physical model with only one degree of internal freedom. As an example, one can cite the lysine ϵ carbons in native MCBP-3. These have an apparent T_1 of 170 ms (NT_1 = 340 ms) and NOE = 2.5. If these carbons were experiencing isotropic reorientation, the T_1 could correspond to τ_c = 0.1 ns or τ_c = 100 ns; however, NOE = 3.0 is predicted for the former

and NOE 1.15 for the latter. On the other hand, in order to accommodate the experimental NOE value with the isotropic model, NT_1 of 70 ms would be necessary, corresponding to an experimental prediction of T_1 = 35 ms which is a factor of 5 smaller than the measured value. A more refined theoretical treatment is clearly needed and is being developed.

Motion of Phenyl Groups

A specific case in which a more rigorous analysis is possible is that of the side-chain phenyl groups. Phenyl groups can undergo reorientation about two different internal bond axes, C_β–C_γ or C_β–C_α, in addition to the isotropic reorientation of the framework for the molecular axes. In order to describe the phenyl group motions three physically distinct correlation times must be considered. Even though the motions described by these correlation times are independent, their effects on relaxation are not. Several simplifications can be made in order to treat this problem. The most important of these is to use the independently measured value of the overall correlation time.

Previous attempts at describing the intramolecular motions of phenyl groups in macromolecules in solution by carbon nuclear resonance have been focused on the model systems of polystyrene (Allerhand and Hailstone, 1972; Schaefer and Natusch, 1972) and poly(γ-benzyl glutamate) (Allerhand et al., 1973a,b). The progression of values of relaxation parameters along the side-chain positions in both polymers indicates that faster motions are present as a carbon becomes further removed from the α-carbon backbone.

The intramolecular motions in polystyrene have been described in terms of relatively rapid local segmental motions. While an isotropic rotational correlation time has been assigned to these rotations on the basis of the T_1 values, a different time is obtained from the NOE measurements from the aliphatic and quaternary phenyl carbons. This situation is analogous to that for amino acid side chains on MCBP where no value of τ_c can account for both the T_1 and the NOE data. We have measured the T_1 and NOE on a 20% solution (w/v) of 25 000 molecular weight polystyrene. No previous NOE measurements have been made on the resolved phenyl ring carbons and the results listed in Table II indicate that there is a differential in NOE among the ring carbon positions. There is no difference among the T_1 values for the positions with bonded protons. The C_4 position has the same value as the aliphatic carbons, which is a smaller value than the $C_{2,6}$ and $C_{3,5}$ positions. This relaxation behavior can be accounted for by an internal rotation occurring about the C_1–C_4 molecular axis. Although a complete analysis of the motions of polystyrene has not been carried out, this differential NOE shows that the relaxation methods employed here are sensitive to both the rate and the angle of internal rotations of phenyl groups.

The phenyl groups of MCBP, which has been denatured by guanidine hydrochloride and heat, may have motions similar to those of the benzyl groups of polystyrene and poly(γ-benzyl glutamate) in the random coil form. The relaxation parameters for the denatured MCBP phenylalanines are T_1 = 200 ms and NOE = 2.3. The NOE value is similar to the 2.5 found for the random coil poly(γ-benzyl glutamate); however, the T_1 is considerably shorter. These values do not correspond to any isotropic rotational correlation time. In the fully denatured protein many complex rapid segmental motions are likely to be present, and the resolution of individual phenyl ring carbons is too low to observe differential effects among different positions.

In the native protein, the phenyl ring carbons with directly bonded hydrogens have a NOE = 1.4, significantly higher than the minimal value observed for the α-carbon atoms of the protein main chain (i.e., for the α carbons, NOE = 1.2). This somewhat unexpected finding for the carbon atoms forming the tightly knit hydrophobic core of the MCBP molecule can only be explained by assuming that the phenylalanine side chains do, in fact, possess relatively rapid intramolecular motion. This motion can be described as follows. The principal axis for rotation of the phenyl rings is taken to be the C_β–C_α axis, with which the C–H vectors form an angle of 60°. From the theoretical plots for τ_1 with θ = 60° superimposed on a 12-ns isotropic correlation time (Figure 3), it can be determined that the experimental relaxation parameters (T = 55 ms, NOE = 1.4) can be simultaneously accounted for only by a single value of the internal rotational correlation time, i.e., 4 ns. There is some uncertainty in this value, because the Woessner model used in the calculation does not precisely represent the kind of hindered rotation that an amino acid side chain is likely to undergo in the interior of the protein. Nor does it make a distinction between oscillations over a limited angular range and complete rotation. However, theoretical analysis of the angular dependence of internal rotations which will be published elsewhere permits the conclusion that *some* additional internal motion, with a rate of the order of, or faster than, the overall rate of rotational diffusion ($\tau_1 \leq 12$ ns) must be assumed to account for the observed values of the relaxation parameters. It is of interest to note that in a recent report internal rotation of MCBP phenylalanine ranges at a rate faster than 10^3 s^{-1} has been inferred from ^1H NMR spectra (Cave et al., 1976).

TABLE II: Polystyrene Relaxation Data.

Carbon Position	T_1 (ms)	NOE
CH_2	50	2.0
CH	90	2.1
C_1		2.0
$C_{2,3}$	110	2.3
C_4	100	2.1

Conformational Change Resulting from the Removal of One of the Ca^{2+} Ions

Native MCBP contains two tightly bound calcium ions, one of which, the EF calcium, possesses solvent water as one of the liganding groups. This solvent exposed metal ion can be easily removed by mild treatment with EGTA (Donato and Martin, 1974; Nelson et al., 1976). The metal ion dependent conformational transition detected previously by several physical methods (Parello et al., 1974; Donato and Martin, 1974) is also seen in the ^{13}C NMR spectra.

The effect of calcium removal on the conformation of MCBP-3 is shown in Figures 4 and 5. Spectral differences are observed in all resonance regions. The largest change following Ca^{2+} removal involves the methyl groups of the alanines, where a change from a homogeneous band is caused by a significant number of resonances being shifted downfield. The large peak from the ϵ carbon of the lysines becomes more intense upon removal of the EF calcium; this can be seen most clearly in the difference spectrum as a substantial negative peak. The relaxation experiments summarized in Table III show that the intensity change is related to an increase in NOE for this peak upon Ca^{2+} removal, a finding that is consistent with some of

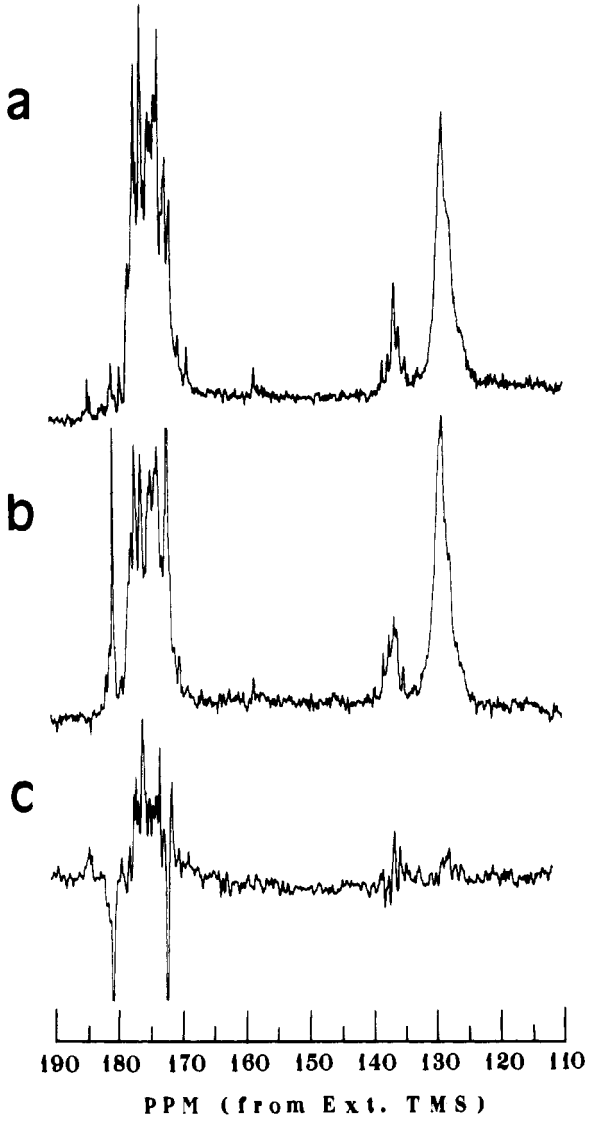

FIGURE 4: Downfield spectral comparison of MCBP-3 with two and one bound calciums. (a) MCBP-3 at a concentration of 20 mM with two bound calcium ions; 50 000 transients were collected. (b) MCBP-3 at a concentration of 14 mM after the removal of the solvent accessible EF calcium by EGTA. The additional large peaks in the spectrum are due to the added chelating agent. Transients (75 000) were collected. (c) Difference plot, obtained by subtracting the normalized free induction decays of b from a.

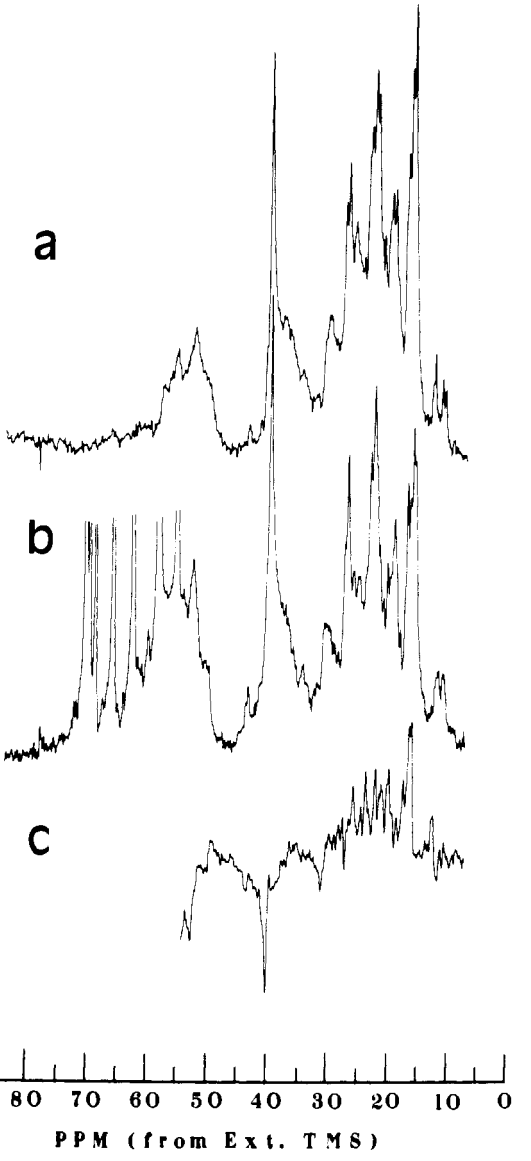

FIGURE 5: Upfield spectral comparison of MCBP-3 with two and one bound calciums. Same experimental conditions as in Figure 4.

TABLE III: NOE and T_1 for Different Amino Acid Side Chains in the Two Conformational States of MCBP.

Amino Acid	Carbon	2 Ca^{2+} NOE	2 Ca^{2+} T_1 (ms)	1 Ca^{2+} NOE	1 Ca^{2+} T_1 (ms)
Ala	β	1.8	215	2.0	215
Asp, Leu, Phe	β	1.3	50	1.3	50
Thr	γ	1.9	130	2.1	130
Lys	δ	1.7	100	2.1	100
Lys	ϵ	2.3	170	2.6	130
α region		1.2	55	1.2	
Phe		1.4	55	1.4	55
Phe	γ	1.0	350	1.0–1.1	450
Carbonyl		1.2		1.2	

the lysine side chains going to a state of greater motional freedom. The δ carbons of the isoleucine residues give similar patterns for both parvalbumin isotypes studied (Figure 1) and the difference spectrum (Figures 4c and 5c) unambiguously shows the changed resonance pattern for MCBP-3 to be due to an upfield shift of a single resonance. This change is indicative of one carbon going to a more highly electron shielded environment upon Ca^{2+} removal.

Changes at individual carbon sites are observed among the well-resolved downfield resonances. The most downfield carboxyl carbon resonance (184.6 ppm) attributed to Glu-81, whose carboxyl group participates in an internal ionic bond with the guandio group of Arg-75, shifts dramatically upfield upon removal of the EF calcium. The chemical shift for this resonance in protein with one bound calcium lies well within the carboxyl–carbonyl resonance envelope. The Arg-75 guanido carbon does not shift following the release of one Ca^{2+} as one might expect. However, it is known that guanido carbons are relatively insensitive to the effects of environmental perturbation.

Significant changes of signal intensities and chemical shifts are observed throughout the entire carbonyl region indicative of some alterations in secondary structure with the removal of the calcium ion. The two upfield shifted carbonyl resonances

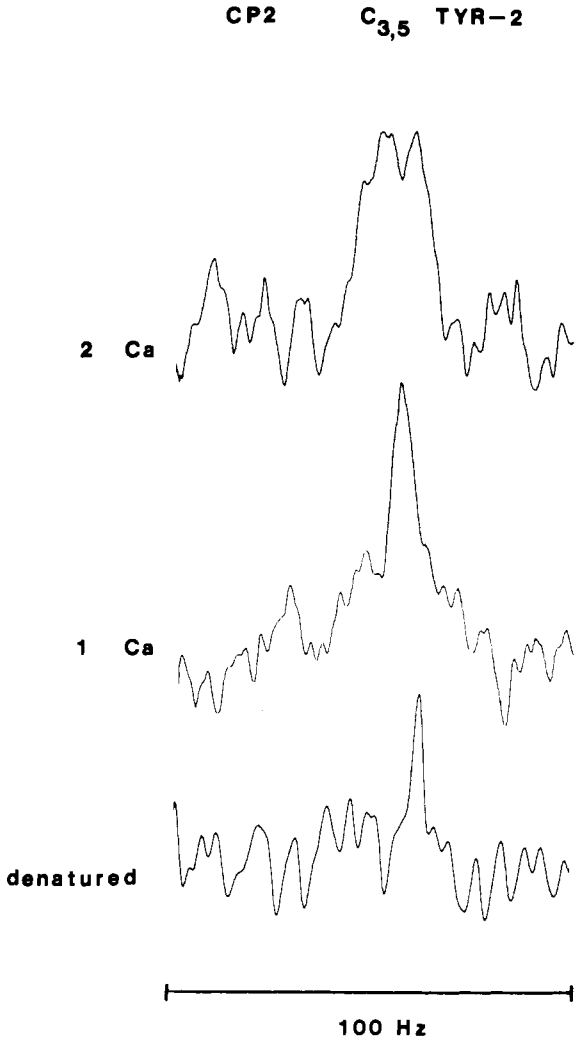

FIGURE 6: Carbon-13 resonance signals of the two C_ϵ tyrosine (Tyr-2) ring carbons of MCBP-2, in the presence of two bound calciums, one bound calcium in the denatured state. Same experimental conditions for MCBP-2 as given in Figure 1.

most probably associated with Phe-57 and Lys-96, assigned on the basis of the predicted effect of calcium coordination on the carbonyl bond lengths, are differentiated by these experiments. The most upfield carbonyl signal is shifted downfield, to a position within the carbonyl envelope, by the removal of the EF calcium. Since the carbonyl group of Lys-96 coordinates calcium in the EF site, the peak at 168.9 ppm in the native protein can be attributed to the carboxyl carbon of Lys-96, and the carbonyl carbon at 170.2 ppm can be attributed by elimination to Phe-57. The differential behavior observed for these two upfield carbonyl carbon resonances is evidence for calcium ion being completely and selectively removed under the conditions employed. Preliminary results on the removal of calcium from MCBP-2 indicate that it is again the most upfield shifted carbonyl signal that undergoes change. The identical behavior of MCBP-2 and MCBP-3 with respect to the primary event of calcium removal from the EF site argues in favor of the carbonyl assignments and the homologous nature of the two calcium binding proteins.

The spectral changes in the aromatic region (100–150 ppm) with the removal of calcium are most easily seen in the difference spectrum (Figure 4c). These changes can be correlated with alterations in the environment of the rings of the phenylalanine residues which are the main constituents of the protein's hydrophobic core. The phenylalanine γ carbons (135–140 ppm) yield a difference pattern indicative of substantial chemical shift changes for both isotypes. In contrast, the resonance band arising from ring carbons with directly bonded hydrogens (125–135 ppm) shows only minor differences between samples for MCBP-3 with one and two bond calciums, in spite of having five times the number of contributing nuclei. Interestingly enough, the observed change in chemical shift is not accompanied by any large changes in the relaxation parameters, of most amino acid side chains as shown in Table III.

The most striking effect of Ca^{2+} removal on the structure of the protein at a point distant from the Ca^{2+} binding site is seen in the spectrum of tyrosine-2 (Figure 6). In native MCBP-2, three of the six ring carbons of Tyr-2 can be resolved in the ^{13}C spectrum. One of these, the ϵ carbon is a doublet. There is no apparent change of the tyrosine ζ carbon as calcium is removed suggesting that there is no large alteration in the environment of the residue; however, the two resonance signals associated with the two ϵ-carbon atoms collapse to a single peak as a result of calcium removal (Figure 6), clearly indicating that the ring is free to rotate about the C_β–C_α axis in the monocalcium, but not in the dicalcium form of the protein. Unfortunately the intensity of the Tyr peak is too low to permit relaxation measurements. However, this finding as well as the finding of an increased NOE for the ϵ groups of lysine clearly indicates that different conformational states of a protein may be characterized not only by differences in the spatial configuration of certain specific amino acid side chains but also by differences in their states of motion.

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Chymotryptic Conversion of Bacterial Membrane ATPase to an Active Form with Modified α Chains and Defective Membrane Binding Properties†

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ABSTRACT: The effect of chymotrypsin on the structure, catalytic activity, and membrane binding properties of the energy transducing ATPase in *Streptococcus faecalis* was examined. Chymotrypsin caused a limited cleavage of the solubilized ATPase producing a fully active protein which migrated as a single faster moving band in gel electrophoresis. The modified ATPase, designated CHY-ATPase, was relatively resistant to further chymotryptic alteration. It retained the full complement of tightly bound nonexchangeable nucleotide present in the native ATPase. The CHY-ATPase contained modified α chains, designated α', which were about 2000 daltons smaller than the native 55 000-dalton α chains. However, no alterations in the other subunits, β, γ, δ, or ϵ, were detected. It appears that the subunit composition of the native ATPase, $\alpha_3\beta_3\,\gamma\,\delta\,\epsilon$, was changed to $\alpha'_3\beta_3\,\gamma\,\delta\,\epsilon$. In contrast to the native ATPase, the CHY-ATPase failed completely to reattach to depleted membranes. We have concluded that short chymotrypsin-sensitive α chain "tails" protrude from the ATPase surface and that these peptide segments are needed for membrane attachment. The δ chain was also shown to be required for attachment, confirming previous work (Abrams, A., Morris, D., and Jensen, C. (1976), *Biochem. Biophys. Res. Commun. 69,* 804). This suggests that the three α chains and the δ chain act together as a device to ensure firm association of the ATPase with the membrane.

The membrane-bound ATPase in the fermentative organism, *Streptococcus faecalis,* serves to link solute accumulation to the hydrolysis of glycolytically generated ATP (Harold et al., 1969; Abrams et al., 1972; Smith and Abrams, 1973). It is believed that the ATPase mediates proton extrusion through the membrane thereby generating an electrochemical potential which then drives specific transmembrane solute movements (Harold, 1972; Simoni and Postma, 1975). The molecular basis of this chemiosmotic coupling mechanism (Mitchell, 1966) is poorly understood at present. To arrive at a better understanding of this energy transducing system, we have sought for a number of years to characterize the structure of the ATPase and the membrane components with which it is associated. Some years ago it was shown that the solubilized *S. faecalis* ATPase is a 385 000-dalton oligomeric protein made up of nonidentical subunits (Abrams, 1965; Abrams and Baron, 1967; Schnebli and Abrams, 1970; Schnebli et al., 1970). A recent assessment of the subunit composition indicates that the enzyme consists of five subunit types with approximate mo-

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