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Binding of Regulatory Nucleotides to Aspartate Transcarbamylase: Nuclear Magnetic Resonance Studies of Selectively Enriched Carbon-13 Regulatory Subunit†

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ABSTRACT: Specifically enriched [γ-^{13}C]phenylalanine, -tyrosine, and -histidine have been biosynthetically incorporated into aspartate transcarbamylase from *Escherichia coli*. These nonperturbing NMR probes have been used to characterize the interaction of the regulatory sites on the enzyme with nucleotide effectors. The C_γ carbons of the three tyrosines and four histidines per regulatory chain give narrow, well-resolved resonances, and the signals from the five phenylalanines per chain are partially resolved in the presence of bound inhibitor. Spectral changes in regulatory subunit were monitored as a function of concentration of the inhibitor, CTP, and the activator, ATP. Three histidine residues responded to ATP and CTP in an identical manner while two phenylalanine residues were sensitive to CTP but not ATP binding. The tyrosine resonances were not perturbed by effectors. The chemical shift response of the single observable histidine resonance to bound nucleotides in the reconstituted enzyme was identical with that observed for isolated regulatory subunit. This histidine spectrum was undisturbed by the *T* to *R* conformational transition of the enzyme. The results suggest that the regulatory subunit experiences minimal rearrangement of tertiary structure on binding effectors and that at least one phenylalanine and one histidine residue are present in the region of the CTP binding site.

Aspartate transcarbamylase (ATCase)[1] (EC 2.1.3.2) from *Escherichia coli* catalyzes the formation of carbamyl-L-aspartate from L-aspartate and carbamyl phosphate, the first committed step in the biosynthesis of pyrimidines (Gerhart, 1970; Schachman, 1972; Jacobsen & Stark, 1973). The enzyme serves as a major control point in this metabolic pathway and is subject to inhibition by CTP and activation by ATP. The 310 000-dalton molecule contains two distinct types of subunit, one type for catalysis and the other for regulation. The native hexameric enzyme is composed of two trimeric catalytic subunits of 100 000 daltons each and three dimeric regulatory subunits of 34 000 daltons. Electron microscopy (Richards & Williams, 1972) and X-ray diffraction studies (Monaco et al., 1978) indicate that the catalytic trimers are not in direct contact but instead are linked via the regulatory dimers. The subunits are separable, and each retains its individual function in the isolated state (Gerhart & Schachman, 1965).

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[1] Abbreviations used: NMR, nuclear magnetic resonance; ATCase, aspartate transcarbamylase; PALA, *N*-(phosphonoacetyl)-L-aspartate.

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ATCase has been the subject of a great deal of research aimed at understanding allosteric regulatory mechanisms. The observed homotropic and heterotropic effects are generally attributed to an equilibrium between two conformationally distinct states of the enzyme. The changes in the quaternary structure of the enzyme promoted by substrate binding have been extensively studied (Schachman, 1972; Howlett et al., 1977). In addition, the interaction of substrates with the catalytic site and the catalytic mechanism are relatively well understood (Jacobsen & Stark, 1973). Considerably less information is available about the regulatory site and the role of the regulatory subunit in mediating the observed conformational changes. In order to obtain specific information concerning the structure of the regulatory subunit and the changes attending nucleotide binding, we have introduced nonperturbing ^{13}C NMR probes into the protein by incorporation of specifically enriched amino acids in vivo. Phenylalanine, tyrosine, and histidine were chosen for study. These residues have been shown to participate in pyrimidine binding in other proteins (Wyckoff et al., 1970; Arnone et al., 1971) and have the added advantage of containing quaternary (nonprotonated) carbons in the side chain. This type of carbon generally exhibits narrow signals which are highly sensitive to local environment (Oldfield et al., 1975; Browne et al., 1976; Otvos & Browne, 1980). The 5 phenylalanines, 3 tyrosines, and 4 histidines per regulatory chain have enabled us to monitor the response of 24 approximately randomly distributed sites (Weber, 1968) in the regulatory subunit.

Materials and Methods

Materials. DL-[γ-^{13}C]Phenylalanine and L-[γ-^{13}C]tyrosine were synthesized from 90% ^{13}C-enriched $BaCO_3$ (Monsanto) as previously described (Moore, 1976). DL-[γ-^{13}C]Histidine was the generous gift of Dr. Elaine M. Earl (Browne et al., 1976; Earl, 1978). CTP, ATP, carbamoylphosphate, and L-aspartic acid were obtained from Sigma Chemical Co. The carbamoyl phosphate was purified by precipitation from 50% ethanol and stored desiccated at −20 °C. PALA was the gift of Dr. Willy Shih. D_2O was 99.8% pure from Bio-rad. L-[^{14}C]Aspartic acid was obtained from New England Nuclear.

Enzyme and Subunits. The enriched amino acids were incorporated singly into overproducing ATCase mutant *E. coli* as previously described (Moore, 1976). No dilution or metabolic degradation of the labeled amino acids was found to occur. ATCase was isolated by using a modified (Moore, 1976) version of the procedure of Gerhard & Holoubek (1967). Regulatory subunit containing two structural zinc ions and catalytic subunit were isolated and reconstituted by using the procedure of Kirschner (Kirschner, 1971; Yang et al., 1978). When necessary, reconstituted protein was purified by passage through Sephadex G-200. Thiol groups were determined by the method of Vanaman & Stark (1970). Enzymatic activity was measured in 0.04 M potassium phosphate, pH 7.0, by a colorimetric procedure (Prescott & Jones, 1969) or by radioassay (Porter et al., 1969). Polyacrylamide gel electrophoresis was conducted on 7% gels with the buffer system of Jovin et al., (1964). Concentrations of pure protein were determined spectrophotometrically by using adsorption coefficients of 0.59, 0.72, and 0.32 cm^2/mg for ATCase, C subunit, and R subunit, respectively (Gerhart & Holoubek, 1967). Filtration of the protein through Millipore filters (0.45-μm pore) preceded each determination.

Preparation of Samples for NMR Measurements. Spectra of regulatory subunit were obtained in D_2O by using a buffer system of 0.01 M potassium phosphate, 50 mM mercaptoethanol, and 2 mM $ZnCl_2$. Spectra of reconstituted ATCase were obtained in H_2O by using a buffer system of 0.1 M phosphate, 10 mM mercaptoethanol, and 2 mM EDTA. Protein was transferred into D_2O by repeated ultrafiltration with an Amicon PM-10 membrane. Specific activity was measured for reconstituted samples. Analytical disc gel electrophoresis was performed on all samples. Measurement of pH was made on a Sargent Welch Model NX pH meter both before and after each spectrum with a Beckman No. 39505 combination electrode. Reported pH values are uncorrected for the deuterium isotope effect at the glass electrode. Titrations with ligand were carried out by adding the ligand directly into the NMR tube by means of a micropipet. The stock ligand solutions (0.2–0.5 M in ligand in 0.1 M potassium phosphate) were freshly prepared and adjusted to the pH of the protein solution. Precise concentrations of ligand solutions were determined from known extinction coefficients. The high concentrations of regulatory subunit used in these studies apparently did not lead to aggregation of the protein, as evidenced by the fact that ^1H NMR spectra (220 MHz) at 7 and 80 mg/mL were identical.

NMR Methods. Carbon-13 NMR spectra were recorded at 25.2 MHz on a Varian XL-100-15 spectrometer equipped with a Nicolet TT-100 Fourier-transform package. A spectral width of 5000 Hz was used with a digital resolution of 2.5 Hz. The samples were contained in 12-mm tubes equipped with Vortex plugs and held at 6 ± 1°. Generally, a 70° pulse and a recycle time approximately equal to the T_1 values for the sample were used. An internal D_2O lock was used for all spectra except for that of ATCase in H_2O buffer. In this case, the spectrometer was locked on external D_2O contained in a coaxial insert. Chemical shifts were measured with internal dioxane as a reference and are reported relative to external Me_4Si. Spin–lattice relaxation times were obtained by the progressive saturation method (Freeman & Hill, 1971). Data were analyzed by using a nonlinear least-squares fit to a single exponential decay. Nuclear Overhauser enhancement (NOE) measurements were made by using the gated decoupling technique (Opella et al., 1974).

Table I: Chemical Shift and Relaxation Parameters for C γ in the Aromatic Residues of Aspartate Transcarbamylase Regulatory Subunit at 25 MHz[a]

residue	chemical shift[b] (ppm)	T_1 (s)[c]	NOE[d]
histidine	131.3	1.24	1.48
	133.3	2.78	1.26
	134.4	3.14	1.18
tyrosine	127.9	1.17	1.21
	129.2	0.94	1.31
	130.6	1.32	1.13
phenylalanine	137.3	2.18	1.28
	137.7	2.08	1.28
	138.2[d]	2.65	1.28

[a] The following chemical shifts are observed for the regulatory subunit at pH 7.1 denatured in 6 mM guanidine hydrochloride: phenylalanine, 137.55; tyrosine, 129.20; histidine, 134.06 ppm. [b] Downfield from external Me_4Si. All solutions were pH 7.1. [c] When more than one determination was made, values shown are averaged. Estimated accuracy ±10%. [d] Downfield shoulder.

Results

The selectively enriched residues in the isolated regulatory subunit give rise to the relatively simple spectra shown in Figure 1. Chemical shifts, T_1, and NOE values for the signals at pH 7.1 are given in Table I. The tyrosine spectrum (Figure 1B) consists of three individual carbon resonances, corresponding to the three tyrosines per regulatory chain. The center resonance has a chemical shift identical with that found

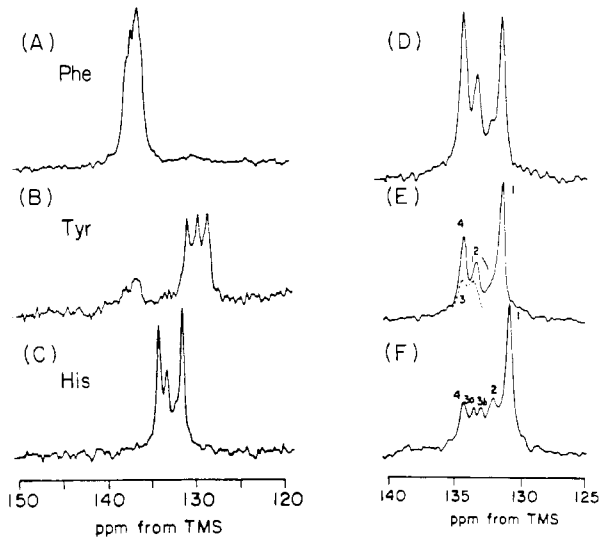

FIGURE 1: ^{13}C NMR spectra of C_γ carbons in the aromatic residues of ATCase regulatory subunit at 25 MHz. Resonance positions are given in ppm downfield from external Me_4Si. Signals are artificially broadened 4 Hz by pretransform exponential filtering. Spectra A–E were taken at pH 7.1. (A) Phenylalanine resonances, 8044 transients. Protein concentration if 1.74 mM. (B) Tyrosine, 12 000 transients, 1.2 mM. (C) Histidine, 6368 transients, 1.87 mM. (D) Histidine, obtained under conditions of gated decoupling and full relaxation, 8000 transients, 1.87 mM. (E) Histidine, 8000 transients, 1.87 mM. (F) Histidine, 8000 transients, 2.1 mM, pH 6.6.

for tyrosine in the protein unfolded in 6 M guanidine hydrochloride. The five phenylalanines per chain (Figure 1A) appear in a broad envelope downfield of the phenylalanine chemical shift in the denatured chain. Regulatory subunit contains four histidines per chain, and four peaks are apparent in Figure 1C. However, integration carried out on a spectrum obtained with gated decoupling and no sensitivity enhancement (Figure 1D) gives a ratio of 3:2:1:2 for the true intensities of the peaks. The resonance position of the histidine quaternary carbon is a function of pH near neutrality. At pH 6.6, the histidine spectrum is resolved into five individual resonances (Figure 1F). The signal intensities under conditions of gated decouplings are 2:1:1:2:2 for the eight histidines per dimer. This is consistent with an asymmetric arrangement of identical chains or with distinct conformational states with a nearly equal equilibrium distribution. Individual pK_a values for the histidines could not be obtained due to the instability and lower solubility of the regulatory subunit at pH values below 6.4. Measurement of resonance position as a function of pH between pH 6.6 and 7.1 (data not shown) results in the assignments at pH 7.1 shown in Figure 1E. As the pH is gradually increased from 6.6 to 7.1, histidine-1 moves downfield, histidine-2 splits into two peaks with half of the intensity titrating downfield, and peaks 3A and 3B move downfield as individual resonances. At pH 7.1 (Figure 1E), 3A has shifted under peak 4, and 3B occurs at about the same position as peak 2A. Histidine-4 remains unperturbed at pH values above 6.6.

The changes in histidine environment accompanying effector binding were studied near pH 7 where the association constants for CTP and ATP have been measured (Gray et al., 1973), and also at lower pH where resonances 3A and 3B are resolved. A typical CTP titration is shown in Figure 2 along with difference spectra from both CTP and ATP binding studies at pH 7.1. When 2 mol of nucleotide have bound to the dimer, the signal from histidine-1 shifts upfield by 0.4 ppm, histidine-2B shows a modest (0.1–0.2 ppm) downfield shift, and a large upfield shift (1.5 ppm) is experienced by histidine-3. The effects of activator and inhibitor are identical, both at pH

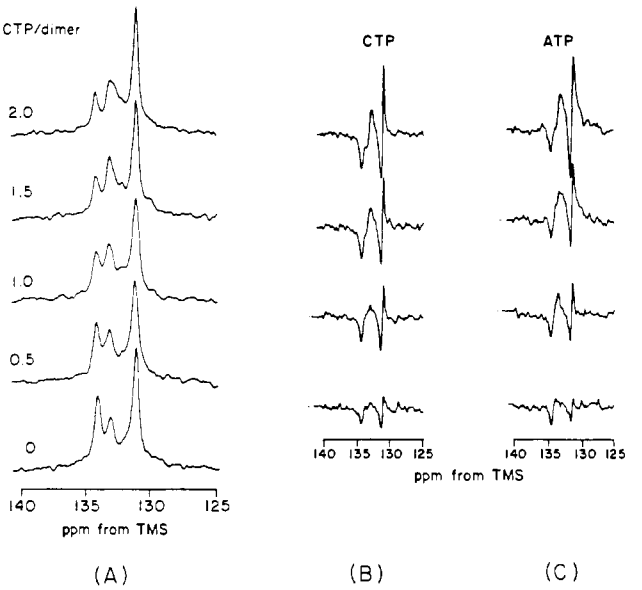

FIGURE 2: ^{13}C spectra (8000 transients) of [γ-^{13}C]histidine regulatory subunit at pH 7.1 as a function of added CTP. (A) Line broadening is 4 Hz. Protein concentration is 1.87 mM. (B) Difference plot of (A) generated by subtracting the spectrum with CTP absent from each of the spectra with CTP present. (C) Difference plot of a similar titration carried out with ATP. Protein concentration is 2.1 mM..

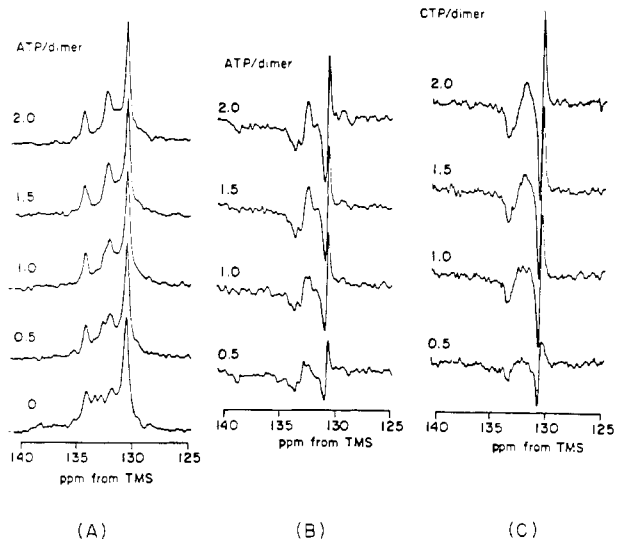

FIGURE 3: ^{13}C spectra (8000 transients) of [γ-^{13}C]histidine regulatory subunit at pH 6.6 as a function of added ATP. (A) Line broadening is 4 Hz. Protein concentration is 1.76 mM. (B) Difference plot of (A). (C) Difference plot of a similar titration carried out with CTP, pH 6.75. Protein concentration is 1.76 mM.

7.1 and at pH 6.6. A typical ATP titration of regulatory subunit is shown in Figure 3. The changes in histidine resonance position which accompany ATP and CTP binding are summarized in Table II.

The identical effects of activator and inhibitor on the histidine residues are in marked contrast to their differential effects on the phenylalanine resonances. It is apparent from Figure 4 that CTP causes major alterations in the phenylalanine environments. The shift of phenylalanine-2 downfield by 0.4 ppm results in partial resolution of resonance 1. Peak 5 experiences a very large (1.6 ppm) downfield shift. However, an analogous titration carried out with ATP showed no change in the resonance position of phenylalanine residues. The sensitivity of the tyrosine environment to ATP and CTP binding was also investigated. At pH 7, no significant changes

Table II: Chemical Shift Changes in Regulatory Subunit on Nucleotide Binding[a]

label	pH[b]	carbon[c]	chemical shift (ppm) no ligand	with ATP	with CTP	Δ ATP	Δ CTP
phenylalanine[d]	7.1	2	137.2	137.2	137.6	0.0	−0.4
		5	137.9	137.9	139.5	0.0	−1.6
histidine	7.1	1	131.4	130.9	131.0	0.5	0.4
		3	134.4	132.9	132.9	1.5	1.5
histidine	6.75	1	130.8		130.5		0.3
		3b	133.2		132.1		1.1
		3a	133.6		132.1		1.5
histidine	6.6	1	130.7	130.4		0.3	
		3b	133.0	132.2		0.8	
		3a	133.5	132.2		1.3	
histidine in ATCase[e]	7.1	1	131.6	131.2	131.2	0.4	0.4

[a] Chemical shift values were obtained from difference spectra at 25 MHz and are reported relative to external Me_4Si. The shifts are accurate to within 0.1 ppm. Resonances not shown in the table displayed no observable shifts except histidine-2b, which was difficult to measure accurately. A negative sign refers to a downfield shift. [b] pH values are uncorrected for the isotope effect at th the glass electrode. [c] As shown in Figure 1. [d] Measurements at 15 MHz gave values identical with those at 25 MHz. [e] Performed in H_2O.

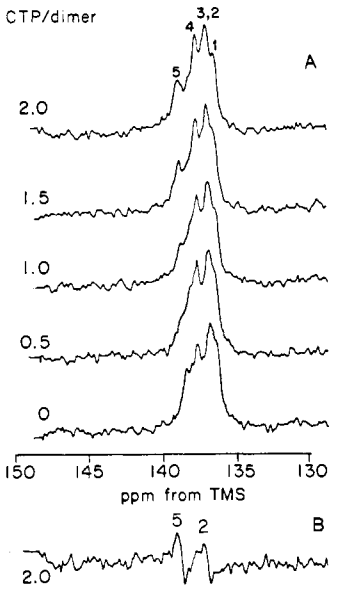

FIGURE 4: ^{13}C spectra of [γ-^{13}C]phenylalanine regulatory subunit at pH 7.1 as a function of added CTP. (A) Line broadening is 2 Hz. Protein concentration is 1.5 mM. (B) Difference plot of unliganded protein and protein saturated with CTP.

from the spectrum seen in Figure 1B were produced by the binding of either nucleotide.

In order to determine whether the changes observed to accompany nucleotide binding in the regulatory subunit also occur in the intact enzyme, ^{13}C-enriched regulatory subunit was reconstituted with unlabeled catalytic trimers. Due to the slow molecular tumbling of the oligomeric complex and the resultant wide lines and long T_1 values, spectra of labeled phenylalanine and tyrosine residues showed broad, featureless resonances of low intensity. Signals from histidines-2, -3, and -4 were also unresolved. However, the relatively fast rate of internal reorientation of histidine-1 largely offset the longer correlation time in the ATCase complex, and its resonance was easily observed (Figure 5). The spectra of the reconstituted enzyme were measured in H_2O since the transfer of the protein to D_2O by repeated ultrafiltration caused aggregation. The resonance position of histidine-1 is shifted 0.2 ppm

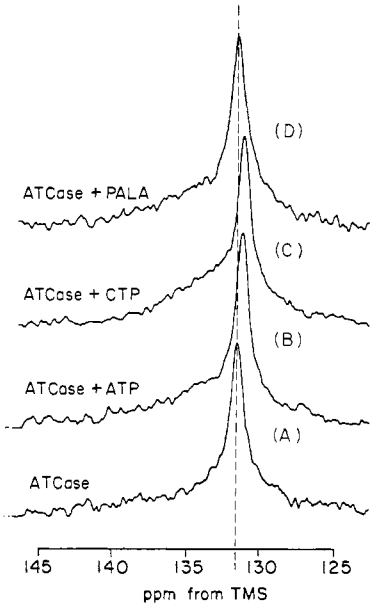

FIGURE 5: ^{13}C spectra of [γ-^{13}C]histidine regulatory subunit reconstituted with unlabeled catalytic subunit. The solvent is H_2O. (A) Unliganded ATCase, 0.68 mM, 40 000 transients. (B) ATCase saturated with ATP, 0.68 mM, 40 000 transients. (C) ATCase saturated with CTP, 0.47 mM, 35 296 transients. (D) ATCase saturated with PALA, 0.54 mM, 47 104 transients.

downfield from its position in isolated regulatory subunit. We attribute this shift largely to the change of solvent (see Discussion). As shown in Figure 5B,C, binding of nucleotides to the ATCase complex causes histidine-1 to shift upfield by the same magnitude as in the isolated regulatory subunit. The change caused by activator and inhibitor is identical. Saturation of the enzyme with the bisubstrate analogue PALA, known to promote a major conformational change (Howlett & Schachman, 1977), has no effect on the position of the histidine-1 resonance (Figure 5D).

Discussion

Measurement of the histidine C_γ chemical shift as a function of pH can be used to assign the tautomeric state of the imidazole ring. The two possible tautomers titrate in opposite directions during imidazolium deprotonation (Reynolds et al., 1973; Oldfield et al., 1975). In the regulatory subunit, the downfield shift of resonances from histidines-1, -2, and -3 with increasing pH is characteristic of the histidine N^τ tautomer, the predominant form in small peptides exposed to aqueous solution (Deslauriers et al., 1975). The position of the C_γ signal from histidine-4 is unchanged over the pH range 6.6–7.1. The failure of histidine-4 to titrate over this range could reflect its location in a buried region of the protein which is inaccessible to solvent. Analysis of relaxation parameters for this residue (Moore, 1976) indicates that it possesses very little internal mobility, suggesting such a buried environment. Although the tautomeric form of histidine-4 cannot be unambiguously assigned, the chemical shift of this resonance is consistent with neutral imidazole in the N^τ tautomer.

The effects of pH on the histidines of the regulatory subunit reveal that histidine-3 gives rise to two resonances of equal intensity. This phenomenon cannot be due to a slow exchange between imidazole and imidazolium forms of the residue because the peaks titrate individually, both in fast exchange. Histidine-2 also appears to give two signals of equal intensity at the higher pH values in the range studied. The observations suggest either two conformationally distinct states of the regulatory dimer with an equilibrium constant close to 1 or

identical chains arranged asymmetrically in the dimer under our conditions. Although additional data are necessary between these alternatives, the observation of magnetic nonequivalence for these residues could have important implications in accounting for the heterogeneity of nucleotide binding found for ATCase. The regulatory dimer exhibits two classes of binding sites for both ATP and CTP (Gray et al., 1973).

In previous studies (Gray et al., 1973; Harrison & Hammes, 1973; London & Schmidt, 1974), the origin of the binding heterogeneity has not been definitively determined although ligand-induced asymmetry (negative cooperativity) has generally been the favored explanation. Our data suggest that regardless of the origin of the inequivalence any differences in conformation or any asymmetry in chain arrangement, whether preexisting or ligand induced, must be relatively small and local in nature. The γ carbons of histidine and tyrosine are extremely sensitive to local environment and provide seven individually resolved and randomly distributed probes per chain. Five of these show no evidence of inequivalence in either the liganded or the unliganded form of the protein. This is consistent with X-ray diffraction studies (Monaco et al., 1978) which show a symmetric arrangement of regulatory chains and very little rearrangement of chain conformation on binding CTP. However, the nucleotide binding site is the one region of the molecule for which the structure has not been determined due to local disorder in the crystals (Monaco et al., 1978). The inequivalence we have observed in perhaps restricted to this region of the molecule.

The binding of ATP or CTP results in identical changes in the histidine resonances. In view of their similar structure and mutually competitive binding (Changeux et al., 1968), several authors have suggested that the ribose and phosphate moieties of the effectors should share the same site and interact with the protein in a similar manner (Gerhardt, 1970; London & Schmidt, 1972). In considering the changes in histidine chemical shift which accompany nucleotide binding, we have attempted to distinguish between local and indirect effects. The changes in chemical shift may reflect the proximity of the bound ligand or a conformational change which alters environments distant from the binding site. The small downfield shift of histidine-2B and the upfield shift of histidine-1 could result from either mechanism. However, the very large shift experienced by histidine-3 (1.5 ppm at pH 7.1) is of the magnitude which might be expected from direct interaction with bound ligand. Further, the small or nonexistent shifts of many of the other reporter groups suggest that a large, general conformational change is absent. In contrast to the histidine-3 resonance, the shift of histidine-1 is most likely due to an indirect effect. Relaxation analysis (Moore, 1976) of histidine-1 indicates relatively fast internal reorientation with a correlation time on the order of 1 ns. Thus, an environment buried within the protein is unlikely. The motional freedom of the ring does not diminish on ligand binding, arguing against a direct interaction with nucleotide. An indirect effect which alters the protonation state of the histidine is, therefore, a more likely origin of the shift. This suggestion is consistent with the demonstration that binding of both ATP and CTP is accompanied by proton uptake (Allewell et al., 1975). Interestingly, these authors pointed out that the pH dependence of proton uptake and the ΔH values of nucleotide binding are suggestive of protonation of histidyl residues.

In contrast to the identical shifts of the histidine resonances produced by effector binding, the spectrum of [γ-^{13}C]-phenylalanine regulatory subunit is perturbed differentially by activator and inhibitor. ATP binding has no effect on phenylalanine resonance positions whereas CTP binding induces downfield shifts in the signals from two residues. The 0.4-ppm downfield shift of phenylalanine-2 could be directly or indirectly induced. The magnitude of the shift is identical with that of the indirect effect seen for the quaternary carbon of phenylalanine-8 in ribonuclease S protein in the presence of the bound inhibitor 2′-CMP (Chaiken et al., 1973). In contrast, the 1.6-ppm downfield shift we observe for phenylalanine-5 is a very large perturbation and, like that for histidine-3, suggests a direct effect. Interestingly, several known pyrimidine binding sites contain the common feature of an aromatic ring which lies 4–5 Å below the pyrimidine ring (Wyckoff et al., 1970; Arnone et al., 1971).

Our ^{13}C NMR data are consistent with the model of the effector binding site proposed by London & Schmidt (1972). In this model, the ribose–phosphate moieties of both effectors are bound in a similar way while the relative positions of amino acid residues interacting with the nucleotide base differ in the ATP and CTP complexes. Our results suggest that the conformations stabilized by the effectors must be similar overall since only 2 of our 12 nuclear reporter groups discriminate between ATP and CTP. Furthermore, the fact that 7 of 12 residues monitored exhibit no spectral perturbations with the binding of either nucleotide suggests that the changes experienced by phenylalanine residues on CTP binding are local. In view of the limited changes in environment we observed for the majority of the aromatic residues upon nucleotide binding, the large shifts exhibited by the C_γ resonances from histidine-3 and phenylalanine-5 suggest that these residues may be located at the nucleotide binding site.

Additional evidence that the changes observed are local is provided by studies of the histidine-labeled regulatory subunit reconstituted with unlabeled catalytic subunit. Assembly of the oligomeric enzyme and transfer from D_2O to H_2O result in a small (0.2 ppm) downfield shift of histidine-1. We tentatively attribute the small shift of histidine-1 to an isotope effect caused by replacement of labile deuterium by hydrogen on histidine nitrogen (Oldfield et al., 1975) and possibly also to a small change in the acid–base equilibrium due to the change of solvent. The high degree of internal mobility of this residue is present in both the isolated subunit and the intact enzyme. Thus, assembly of the oligomeric complex does not significantly alter the environment of histidine-1. Moreover, the changes in the position of the histidine-1 resonance which accompany effector binding to the intact enzyme are the same as those observed for the isolated subunit (Figure 5). Remarkably, the binding of PALA does not alter the magnetic environment of histidine-1 or its mobility, even though this ligand promotes a very large change in overall protein conformation (Collins & Stark, 1971; Howlett & Schachman, 1977) and induces shifts in both tyrosine and histidine resonances in the catalytic subunit (Moore, 1976). The fact that the *T* to *R* transition of the molecule does not disturb the environment of histidine-1 again suggests that the changes in this residue which accompany nucleotide binding are local ones. Our observation that certain regions of the regulatory subunit are unaltered in the major conformational change of ATCase is consistent with the structural similarity of activator and inhibitor. Allowing the effector binding site to remain largely undisturbed by the allosteric transition permits the enzyme to discriminate between activator and inhibitor by restricting changes of conformation accompanying effector binding to a regulatory subsite located near the nitrogenous base. Other structurally similar molecules in the cell which are capable of weak binding to the effector site might have

equal affinity for both states of the protein, and, if so, they would not disturb the conformational equilibrium necessary for metabolic control.

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