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Nuclear Magnetic Resonance Studies of the Role of Histidine Residues at the Active Site of Rabbit Muscle Creatine Kinase†

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ABSTRACT: Fourier transform proton NMR studies at 250 MHz using 16-bit A/D conversion allowed detection of six imidazole C-2 proton resonances and one imidazole C-4 proton resonance of the 16 histidine residues per subunit of rabbit muscle creatine kinase (M_r 82 000). Titrations measuring their chemical shifts as a function of pH* yielded pK' values of 7.0, 7.1, 5.9, and 5.2 for His-2, -3, -4, and -6, respectively, and permitted the assignment of the C-4 resonance to His-3. The pK' of His-2 was unaffected by creatine but was increased by 0.6–0.7 unit on saturation of the enzyme with the phosphorylated substrate phosphocreatine or MgATP, in quantitative agreement with the results of a pH–rate study of creatine kinase [Cook, P. F., Kenyon, G. L., & Cleland, W. W (1981) *Biochemistry 20*, 1204], indicating that His-2 is the general acid/base catalyst which deprotonates the guanidinium group of creatine as it is phosphorylated by MgATP. The pK' values of His-4 and His-6, while too low to fit the kinetic data as the general acid/base catalyst, also increased in response to the binding of phosphorylated substrates. The pK' value of His-3 was not greatly altered by substrate binding, suggesting that His-3 is not directly involved in catalysis. Titrations of creatine kinase with substrates at constant pH* (6.8) monitoring the chemical shifts of His-2 or His-6 yielded dissociation constants for phosphocreatine and MgADP consistent with those derived from kinetic data, indicating active-site binding. The dissociation constant of MgATP so determined agrees with the results of other binding studies but is an order of magnitude lower than kinetically determined K_D values, suggesting a steady-state random kinetic scheme in the forward direction. The exchange rate of MgADP from its creatine kinase complex (350 s^{-1}) determined by NMR line broadening is 5-fold greater than k_{cat}, indicating a kinetically competent E·ADP·Mg^{2+} complex. Direct evidence for the presence of His-2, His-3, and His-6 at or near the active site was provided by the paramagnetic effects of the substrate analogue β,γ-bidentate Cr^{3+}ATP on the longitudinal and transverse relaxation rates of their imidazole protons. The longitudinal relaxation rates yielded distances of 12 Å from Cr^{3+} to the C-2 protons of His-2 and His-6, consistent with His-2 functioning as the general acid/base catalyst and with His-6 interacting electrostatically with the substrates. His-3 is somewhat farther from Cr^{3+}ATP and is so positioned that its C-4 proton is oriented toward the Cr^{3+}, at a distance of 14 Å. His-4 is too far from the Cr^{3+}ATP (≥18 Å) for a paramagnetic effect to be observed, suggesting that the increases in its pK' values induced by the binding of substrates result from indirect conformational effects.

Creatine kinase is a dimeric enzyme (M_r 82 000) which catalyzes the reversible phosphorylation of creatine by Mg^{2+}ATP. While much information exists on the kinetic mechanism (Morrison & James, 1965; Morrison & Cleland, 1966; Schimerlik & Cleland, 1973; Hammes & Hurst, 1969), the ligands of the divalent cation activator (Reed & Leyh, 1980), and the nature of certain amino acid residues at or near the active site, less is known about the precise roles of these residues in substrate binding and catalysis.

A sulfhydryl group, implicated in the catalytic mechanism as a result of complete enzyme inactivation by iodoacetamide (Watts & Rabin, 1962), was found to be nonessential by the use of the smaller sulfhydryl reagent, methyl methanethiosulfonate (Smith & Kenyon, 1974). At the nucleotide binding site, an arginine residue was detected by chemical modification studies with diacetyl (Borders & Riordan, 1975) and by nuclear Overhauser (NOE)[1] studies of the adenine protons of

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0006-2960/81/0420-6155$01.25/0

ADP (James, 1976; Vasak et al., 1979). NOE studies and quenching of the protein fluorescence by nucleotides also implicated one or more aromatic residues, possibly tryptophan, at the nucleotide binding site (Vasak et al., 1979). At the creatine binding site, evidence for a carboxylate residue has been obtained by the effect of pH on the kinetic parameters of creatine and phosphocreatine (Cook et al., 1981) and by affinity labeling with epoxycreatine (Marletta & Kenyon, 1979). This carboxylate group was postulated to localize the positive charge of the guanidinium group of creatine via an ionic interaction (Cook et al., 1981). At the reaction center, a lysine has been suggested to interact with the transferred phosphoryl group on the basis of NOE measurements of the transition state analogue complex enzyme·MgADP·formate·creatine (James & Cohn, 1974). From studies of the effect of pH on rate, Cook et al. (1981) have suggested a histidine to function at the reaction center as a general acid–base catalyst to protonate phosphocreatine and to deprotonate creatine in the course of the reversible phosphoryl transfer. Chemical modification with diethyl pyrocarbonate had previously implicated one or more histidines in catalysis (Pradel & Kassab, 1968; Clarke & Price, 1979).

Because of several possible candidates for the general acid/base catalyst on creatine kinase and because of the widely recognized problems of assigning pK values to specific residues in pH–rate studies (Frey et al., 1971; Kokesh & Westheimer, 1971) an independent study of the catalytic role of histidines in creatine kinase was undertaken. We have recently shown that the use of 16-bit analogue to digital conversion in the collection of FT NMR data significantly improves the signal to noise ratio in spectra of high molecular weight proteins (Meshitsuka et al., 1981a). We report here a 250-MHz proton NMR study, with 16-bit A/D conversion, of the aromatic region of creatine kinase. Of the 16 histidine residues per subunit (Watts, 1973), six C-2 proton resonances and one C-4proton resonance were resolved and their behavior studied as a function of pH and substrate concentration and in response to the stable paramagnetic metal–nucleotide complex Cr^{3+}ATP. From these observations, three histidine residues were shown to be at or near the active site.

Experimental Procedures

Materials. Phosphocreatine, creatine, ADP, ATP, and Pipes[1] were obtained from Sigma. Hexokinase, lactate dehydrogenase, pyruvate kinase, and glucose-6-phosphate dehydrogenase were purchased from Boehringer Mannheim. $C^2H_3COO^2H$ and KO^2H were purchased from Stohler Isotope, and 99.85% ^2H_2O was obtained from Norell Chemical Co. All other compounds were of the highest purity available commercially.

Methods. Enzyme Preparation and Assay. Creatine kinase was purified from rabbit muscle by the method of Kuby et al. (1954) and stored in 10 mM glycine buffer, pH 9.0. The enzyme was found to have a specific activity of approximately 60 IU/mg when assayed by using the coupled pyruvate kinase and lactate dehydrogenase assay (Tanzer & Gilvarg, 1959) and was shown to be homogeneous on 18% $NaDodSO_4$–polyacrylamide gels (Laemmli, 1970). Creatine kinase concentrations were determined from the absorbance at 280 nm by using the extinction coefficient $A^{1\%}_{cm} = 8.8$ (Noda et al. 1954) and a molecular weight of 82000 for the dimeric enzyme (Yue et al., 1967). The enzyme was deuterated, by repeated concentration and dilution in 1 mM K^+Pipes buffer, pH* 7.5, in ^2H_2O in a 10-mL Amicon cell using an Amicon XM 50 membrane and 15 psi of nitrogen.

Substrates and Analogue. The concentration of P-creatine was determined spectrophotometrically by using a coupled assay containing creatine kinase, hexokinase, and glucose-6-phosphate dehydrogenase (Kuby et al., 1954). Possible contaminants in P-creatine and the adenine nucleotides ATP and ADP were checked by thin-layer chromatography on PEI-cellulose (Baker-flex) sheets in 1 M LiCl. Several batches of Na_2ADP were found to contain small amounts (~8%) of AMP. The racemic, stable paramagnetic metal nucleotide complex β,γ-bidentate Cr^{3+}ATP was prepared by method B of Dunaway-Mariano & Cleland (1980).

Magnetic Resonance Methods. ^{1}H NMR spectra at 250 MHz of 0.7–1.6 mM creatine kinase sites in ^2H_2O solution containing 1 mM K^+Pipes were obtained with a Bruker WM 250 pulse FT spectrometer at 25 °C. At least 256 transients were obtained over 8192 data points by using a 16-bit analogue to digital converter and a sweep width of 3000 Hz. A line broadening of 2 Hz was applied to all spectra. Chemical shifts were calculated from the external reference DSS.[1] Selective proton irradiation was used to suppress the residual water signal. Longitudinal relaxation rates ($1/T_1$) were determined by using a 180°–τ–90° pulse sequence (Carr & Purcell, 1954) at 250 and 360 MHz on a Bruker WM 250 and a Bruker WH 180/360 spectrometer, respectively, at 25 °C. Transverse relaxation rates ($1/T_2$) were calculated from line-width measurements at half-height ($\Delta\nu$) by using the relation $1/T_2 = \pi\Delta\nu$. After prolonged experiments (≥24 h), the deuterated enzyme was found to retain at least 70% of its original activity.

Paramagnetic effects of Cr^{3+}ATP on the longitudinal ($1/fT_{1p}$) and transverse ($1/fT_{2p}$) relaxation rates of the histidine C-2 protons of creatine kinase were used to obtain Cr^{3+} to proton distances and lower limit Cr^{3+}ATP exchange rates, respectively, by using the general theory reviewed elsewhere (Mildvan & Gupta, 1978; Mildvan et al., 1980). Diamagnetic effects of creatine kinase on the transverse relaxation rates ($1/fT_{2D}$) of the protons of MgADP were used to estimate the exchange rate of MgADP by using the theory extensively reviewed elsewhere (Jardetzky, 1964; Lanir & Navon, 1971).

^{1}H NMR spectra of creatine kinase in the absence and presence of substrates were obtained over the pH* range 4.6–9.3. pH* measurements were made at 25 °C by using a Radiometer pH meter and an Ingold electrode calibrated with two standard buffers. The pH* values given are the actual meter readings and have not been corrected for the deuterium isotope effect at the glass electrode, and pK' refers to the pK_A of the histidine determined in ^2H_2O solution. Histidine pK' values determined in ^2H_2O from uncorrected glass electrode readings agree well with those determined in ^1H_2O (Meadows, 1972), because the isotope effect on pK values is equal and opposite to the glass electrode effect (Meadows, 1972; Markley, 1973). Adjustment of pH* in titrations was made by adding either 0.1 M KO^2H or 0.1 M $C^2H_3COO^2H$ with a micropipet. The pH of each solution was measured before and after the NMR spectrum was taken, and pH* readings were found to agree within 0.02 pH* unit. When a small amount of protein precipitated after pH adjustment, it was removed by centrifugation in a Fisher Model 59 centrifuge. Back-titration of the enzyme to pH* 6.9 from either the acidic extreme (pH* 4.6) or the alkaline extreme (pH* 9.3) restored the initial spectrum. The observed histidine

[1] Abbreviations used: Pipes, piperazine-N,N'-bis(2-ethanesulfonic acid); DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; $NaDodSO_4$, sodium dodecyl sulfate; pH*, uncorrected pH meter reading in ^2H_2O solutions; pK', pK_A values of histidine residues determined in ^2H_2O solutions; NOE, nuclear Overhauser effect.

Table I: Least-Squares Analysis of pH Titration Data for Histidine Residues of Creatine Kinase without Ligands [a]

His [a]	pK'		n [b]	chemical shift (ppm) [c]		
	$n = 1$	n fitted		δH^+ [d]	δH^0 [e]	$\Delta\delta H^+$, H^0
2	6.98 ± 0.05	6.98 ± 0.03	0.98 ± 0.07	8.61 ± 0.02	7.73 ± 0.02	0.88
3	7.10 ± 0.05	7.10 ± 0.05	0.99 ± 0.03	8.58 ± 0.03	7.62 ± 0.03	0.96
4	5.92 ± 0.03	5.92 ± 0.04	0.99 ± 0.04	8.82 ± 0.03	7.72 ± 0.01	1.10
6	5.36 ± 0.04	5.23 ± 0.09	0.88 ± 0.09	8.79 ± 0.09	7.52 ± 0.01	1.27
3' [f]	6.83 ± 0.13	6.85 ± 0.14	1.06 ± 0.11	7.05 ± 0.05	6.53 ± 0.05	0.52

[a] His-1 is detectable only between pH* values 6.4 and 7.1 and broadens outside this range while His-5 fails to titrate between pH* 6.2 and 7.0 and broadens outside of this pH* range. [b] Hill coefficient. [c] From external DSS. [d] Chemical shift of protonated histidine residue. [e] Chemical shift of deprotonated histidine residue. [f] Assigned as the C-4 proton of His-3 from the data of Tables III and IV (see text).

chemical shifts as a function of pH* (δ_{obsd}) were fitted by a nonlinear least-squares program to the Hill equation

$$\frac{\delta H^+ - \delta_{obsd}}{\delta H^+ - \delta H^0} = \frac{K_a{}^n}{K_a{}^n + [^2H^+]^n} \quad (1)$$

in which K_a is the dissociation constant of the histidine, $[^2H^+]$ is the hydronium ion concentration determined from the pH* measurements, n is the Hill coefficient, δH^+ is the chemical shift of the fully protonated histidine, and δH^0 the chemical shift of the deprotonated histidine. Both four parameter fits (K_a, δH^+, δH^0, and n) and three parameter fits assuming $n = 1$ were obtained.

The titration of creatine kinase with either P-creatine, MgATP, or MgADP was found to produce downfield chemical shifts of certain histidine resonances. The observed chemical shift of the histidine C-2 proton (δ_{obsd}) is a weighted average of the chemical shifts of the free enzyme (δ_0) and the enzyme–substrate complex (δ_b). In eq 2 $[E_0]$ is the concentration

$$\delta_{obsd} = \frac{\delta_0[E_0] + \delta_b[E_b]}{[E_0] + [E_b]} \quad (2)$$

of free enzyme sites and $[E_b]$ that of the enzyme–substrate complex. The sum of $[E_0]$ and $[E_b]$ is equal to the total enzyme site concentration. When eq 2 is used to evaluate $[E_0]$ and $[E_b]$, the dissociation constant (K_D) of the enzyme–substrate complex is calculated from

$$K_D = \frac{[E_0]([S_t] - [E_b])}{[E_b]} \quad (3)$$

where $[S_t]$ is the total substrate concentration and $[S_t] - [E_b] = [S_f]$, the free substrate concentration. Dissociation constants (K_D) were calculated from titration data by using a nonlinear least-squares computer program.

Results and Discussion

Effects of pH on the Histidine Resonances of Creatine Kinase.* Typical 250-MHz proton NMR spectra of the aromatic region of creatine kinase shown in Figures 1, 3, 5, and 6 detect the C-2 proton resonances of 6 of the 16 histidine residues per subunit. For future reference, the six histidine C-2 resonances are labeled, in order from low to high field, as His-1 through His-6. The line widths of the observable histidines at pH* 6.80 range from 6–10 Hz for His-2, -3, -4, and -6 to 28 Hz for His-1. That of His-5, while broad, is not easily measurable. The line widths of His-1 and -5 further increase at pH* values below 6.4 and above 7.1 (Figure 1). The differences in chemical shift reflect differing environments, and the differences in line width probably reflect differing relative mobilities of the histidines in the protein molecule. The other ten histidine resonances presumably are too broad for detection. Each of the six resonances is assigned to one histidine C-2 proton since under all conditions tested, including pH* variation, the effect of substrates, and in T_1 measurements, each resonance behaves as a single species. Integration of fully relaxed spectra at pH* 6.80, both by computer and by cutting out and weighing of the peaks, indicates comparable areas to within ±20% of the six C-2 proton resonances and of one upfield C-4 proton resonance.

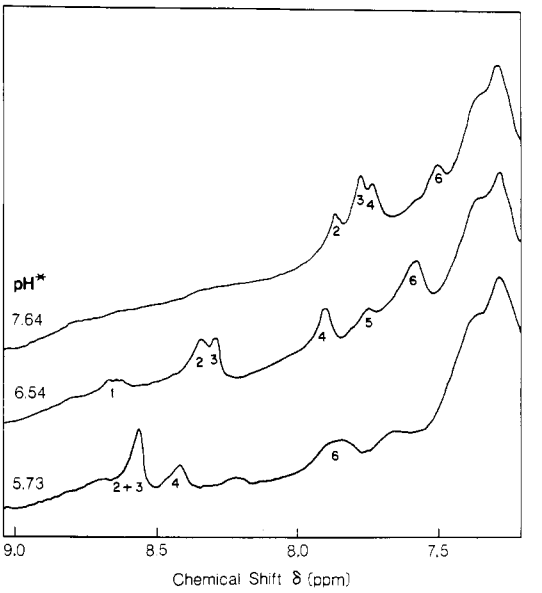

FIGURE 1: Representative NMR spectra showing the pH* dependence of the histidine proton resonances of creatine kinase at 250 MHz. The sample contained 1.2 mM creatine kinase sites in 1 mM K^+Pipes buffer in ^2H_2O in a total volume of 0.4 mL. NMR spectra were obtained at 250 MHz by using 256 transients with 8192 data points, an acquisition time of 1.6 s, spectral width of 3000 Hz, a line broadening of 2.0 Hz, quadrature phase detection, no delay time, 16-bit A/D conversion, a 75° pulse, and saturation by preirradiation of the H_2O signal. $T = 25$ °C. The numbers 1–6 correspond to the histidine C-2 protons of His-1 to His-6.

Representative NMR spectra of the histidine resonances of the free enzyme at several pH* values are shown in Figure 1, and histidine titration curves obtained from such spectra are shown in Figure 2A. Changes in the chemical shifts (δ) with pH* could be fitted with high precision to theoretical titration curves on the basis of eq 1 (Figure 2A, Table I) to yield typical histidine pK' values, end points (δH^+, δH^0) for each titration, and Hill coefficients close to unity for four of the six observable histidine resonances. His-1 broadened beyond detection at pH* values outside of the range 6.4–7.1, and His-5 did not titrate between pH* 6.2 and 7.0 and became undetectably broad outside of this range, precluding a determination of their pK' values.

Only one C-4 proton resonance was observed at higher field (6.87 ppm at pH* 6.54, not shown), the pK' of which is similar to that of both His-2 and His-3 (Table I). On the basis of the agreement of its pK' only with that of His-3 in the phosphocreatine and MgATP complexes (see below), it was assigned as the C-4 proton of His-3. The overall change in

Table II: Least-Squares Analysis of pH Titration Data for the C-2 Protons of the Histidine Residues of Creatine Kinase in the Presence of Creatine

	pK'			chemical shift (ppm)		
His	$n = 1$	n fitted	n	δH^+	δH^0	$\Delta\delta H^+$, H^0
2	6.98 ± 0.04	6.98 ± 0.03	1.01 ± 0.04	8.62 ± 0.01	7.71 ± 0.01	0.91
3	6.85 ± 0.02	6.85 ± 0.02	1.00 ± 0.03	8.62 ± 0.01	7.65 ± 0.01	0.97
4	5.92 ± 0.03	5.96 ± 0.04	1.09 ± 0.04	8.81 ± 0.02	7.72 ± 0.01	1.09
6	5.41 ± 0.08	5.41 ± 0.08	1.00 ± 0.09	8.66 ± 0.08	7.50 ± 0.02	1.16

Table III: Least-Squares Analysis of pH Titration Data for Histidine Residues of Creatine Kinase in the Presence of P-Creatine

	pK'			chemical shift (ppm)		
His	$n = 1$	n fitted	n	δH^+	δH^0	$\Delta\delta H^+$, H^0
2	7.61 ± 0.02	7.64 ± 0.03	0.91 ± 0.05	8.61 ± 0.01	7.69 ± 0.02	0.92
3	7.18 ± 0.02	7.18 ± 0.03	0.94 ± 0.04	8.62 ± 0.01	7.64 ± 0.01	0.98
4	6.33 ± 0.04	6.36 ± 0.04	1.15 ± 0.08	8.65 ± 0.03	7.73 ± 0.01	0.92
6	5.88 ± 0.05	5.84 ± 0.05	0.93 ± 0.06	8.63 ± 0.04	7.53 ± 0.01	1.10
3′ [a]	7.19 ± 0.15	7.12 ± 0.16	1.23 ± 0.40	6.88 ± 0.03	6.44 ± 0.08	0.44

[a] Assigned as the C-4 proton of His-3.

Table IV: Least-Squares Analysis of pH Titration Data for Histidine Residues of Creatine Kinase in the Presence of MgATP

	pK'			chemical shift (ppm)		
His	$n = 1$	n fitted	n	δH^+	δH^0	$\Delta\delta H^+$, H^0
2	7.58 ± 0.04	7.58 ± 0.03	1.14 ± 0.09	8.71 ± 0.01	7.61 ± 0.02	1.00
3	7.10 ± 0.03	7.08 ± 0.03	0.87 ± 0.05	8.63 ± 0.04	7.66 ± 0.03	0.96
4	6.23 ± 0.07	6.21 ± 0.08	0.97 ± 0.08	8.65 ± 0.06	7.71 ± 0.02	0.94
6	5.61 ± 0.07	5.65 ± 0.05	1.15 ± 0.13	8.66 ± 0.06	7.66 ± 0.01	1.00
3′ [a]	7.13 ± 0.02	7.11 ± 0.02	0.83 ± 0.03	7.03 ± 0.01	6.46 ± 0.01	0.57

[a] Assigned as the C-4 proton of His-3.

Table V: Least-Squares Analysis of pH Titration Data for the C-2 Protons of the Histidine Residues of Creatine Kinase in the Presence of MgADP

	pK'			chemical shift (ppm)		
His [a]	$n = 1$	n fitted	n	δH^+	δH^0	$\Delta\delta H^+$, H^0
3	6.83 ± 0.11	6.92 ± 0.03	1.93	8.67	7.71	0.96
4	6.43 ± 0.09	6.41 ± 0.10	0.75	8.61	7.58	1.03
6	5.27 ± 0.10	5.26 ± 0.24	1.67	8.69	7.70	0.99

[a] In the presence of MgADP, the His-2 resonance was too broad for determination of its titration parameters.

chemical shift of this resonance (0.52 ppm) is typical behavior for histidine C-4 protons (Meadows, 1972; Markley, 1973).

Effects of Substrates on the Histidine Titrations. Similar pH* titrations were performed on creatine kinase in the presence of saturating concentrations of the substrates creatine (Figure 2B, Table II), phosphocreatine (Figure 2C, Table III), and MgATP (Figure 2D, Table IV). In each of these substrate complexes, the Hill coefficients remained near unity, and the total changes in chemical shift ($\Delta\delta H^+$, H^0) remained approximately 1 ppm (Figure 2, Tables I–IV), which is typical behavior for the C-2 protons of histidines (Meadows, 1972; Markley, 1973). Analysis of the pH* titration of the single histidine C-4 proton in the enzyme·phosphocreatine (Table III) and enzyme·ATP·Mg^{2+} complexes (Table IV) yielded pK' values in good agreement with those obtained by monitoring the C-2 proton of His-3 but not of His-2. Hence the C-4 proton is assigned to His-3. Further evidence in support of this assignment is that the substrates phosphocreatine, MgATP, and MgADP, which broaden the C-2 proton resonances of His-2 and His-6, have little or no effect on the line width of the C-2 proton resonance of His-3 or of the C-4 resonance.

Saturation of the enzyme with the substrate MgADP caused extensive broadening of the His-2 resonance, preventing the determination of its pK', and increased the Hill coefficients of His-3 and -6 to values approaching 2 in pH* titrations (Table V), suggesting conformational changes in the enzyme·ADP·Mg complex coupled to the protonation and deprotonation of His-3 and His-6. Evidence for more than one conformation of the enzyme·ADP·Mg^{2+} complex has previously been obtained by several spectroscopic and kinetic methods (Lui & Cunningham, 1966; Hammes & Hurst, 1969; Rao & Cohn, 1981).

The effects of the substrates on the pK' values are summarized in Table VI. While creatine had no significant effects on the pK' values of His-2, -4, and -6 and produced only a

Table VI: Changes in pK' of Histidine Residues on Creatine Kinase in the Presence of Substrates [a]

histidine	pK'	(E·creatine) − (E)	(E·P-creatine) − (E)	(E·P-creatine) − (E·creatine)	(E·ATP·Mg^{2+}) − (E)	(E·ATP·Mg^{2+}) − (E·ADP·Mg^{2+})
2	6.98 ± 0.03	≤0.04	0.66 ± 0.04	0.66 ± 0.04	0.60 ± 0.04	
3	7.10 ± 0.05	−0.25 ± 0.04	0.08 ± 0.06	0.33 ± 0.04	−0.02 ± 0.06	0.16 ± 0.04
4	5.92 ± 0.04	0.04 ± 0.05	0.44 ± 0.06	0.40 ± 0.06	0.29 ± 0.09	−0.20 ± 0.13
6	5.23 ± 0.09	0.18 ± 0.12	0.61 ± 0.10	0.43 ± 0.09	0.42 ± 0.10	0.39 ± 0.24
kinetics [b]	7.2 ± 0.3		0.67 ± 0.30			

[a] From the data of Tables I–V. [b] From Cook et al. (1981). Saturating levels of MgADP were present.

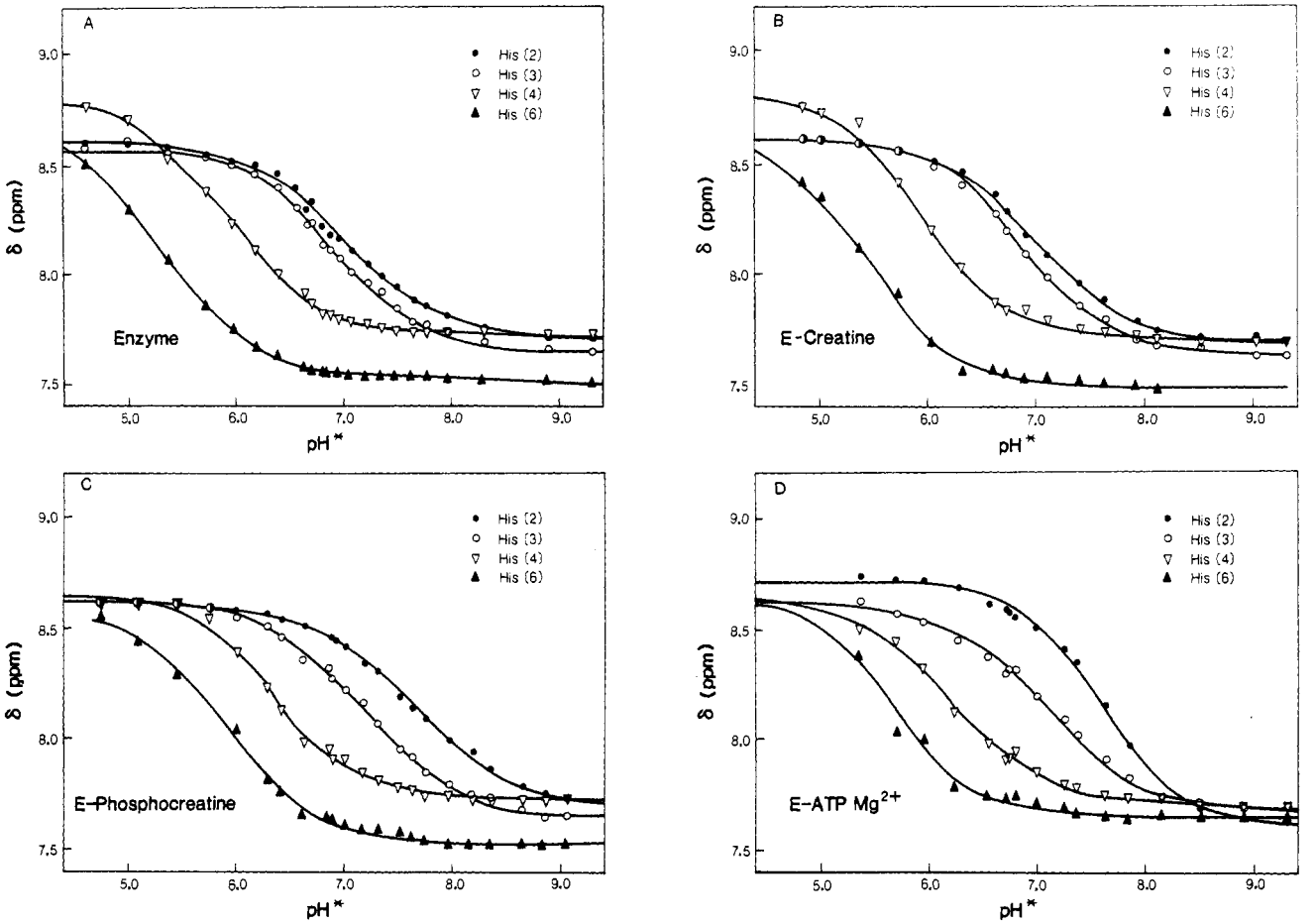

FIGURE 2: Titration curves showing the effect of pH* on the chemical shifts of the histidine C-2 proton resonances of creatine kinase. (A) Titration of the histidine resonances of 1.20 mM creatine kinase sites. (B) Titration of 1.37 mM creatine kinase sites in the presence of 82.6 mM creatine. (C) titration of 1.25 mM creatine kinase sites in the presence of 63.4 mM P-creatine. (D) Titration of 0.82 mM creatine kinase sites in the presence of 3.86 mM MgATP. The symbols refer to His-2 (●), His-3 (○), His-4 (▽), and His-6 (▲). The theoretical curves were fit to the data by a nonlinear least-squares analysis assuming the four parameters (pK', n, δH^+, and δH^0) given in Tables I–IV. NMR spectra were obtained under the conditions described in Figure 1.

small (0.25 unit) decrease in the pK' of His-3, phosphocreatine significantly increased the pK' values of His-2, -4, and -6. The differences between the histidine pK' values in the creatine and phosphocreatine complexes indicate that the presence of the transferable phosphoryl group on the substrate causes the largest increase (0.66 unit) in the pK' of His-2 (Table VI). MgATP, which also has the transferable phosphoryl group, induces a comparable 0.6 unit increase in the pK' of His-2 and smaller effects on the pK' values of the other residues, suggesting that His-2 may be at or near the site of phosphoryl transfer. The pK' value of His-2 of 6.98 and the 0.66 unit increase in this pK' induced by the presence of phosphocreatine (Table VI) are in quantitative agreement with the results of a kinetic study of the creatine kinase reaction as a function of pH (Cook et al., 1981) which detected a pK of 7.2 ± 0.3 in V_{max}/K_M (phosphocreatine), i.e., on the free enzyme, and an increase in this pK of 0.67 unit in V_{max}, i.e., in the enzyme–phosphocreatine complex. This agreement between the NMR and the kinetic studies supports the presence of His-2 at the active site, probably functioning as the general acid/base catalyst suggested by Cook et al. (1981). Modification of one or more histidines on creatine kinase with diethyl pyrocarbonate led to inactivation, and substrates protected the enzyme against such inactivation (Pradel & Kassab, 1968; Clarke & Price, 1979). It is reasonable to suggest that the modification of His-2 would result in inactivation.

Although the pK' values of His-4 and -6 are far too low to fit the kinetic data as the general acid/base catalyst, these pK' values also increase significantly in response to the binding of phosphorylated substrates (Table VI), suggesting that they might also be at or near the active site. This will be shown to be the case for His-6 but not for His-4 by distance measurements (see below). Phosphocreatine produced only a small change and MgATP produced no change in the pK' of His-3, as detected by monitoring the C-2 proton (Tables III, IV, and VI), suggesting that His-3 is not directly involved in catalysis.

Titration of Creatine Kinase with Substrates. Because of their effects on pK' values (Table VI) and on other parameters of the histidine titrations (Figure 2), substrates should alter the aromatic region of the NMR spectrum of creatine kinase at constant pH*. Such effects were used in substrate titrations to test independently whether the pK' changes of the histidines were active-site phenomena.

Titration of the enzyme with phosphocreatine at pH* 6.80 caused a progressive downfield shift of the His-2 resonance (Figure 3) which could be well fit by a calculated titration curve (Figure 4A) obtained by nonlinear least-squares analysis assuming 1.0 binding site per subunit for phosphocreatine with a dissociation constant of 8.7 ± 0.1 mM (Table VII). The dissociation constant so obtained is consistent with the range of K_D values for phosphocreatine obtained by steady-state kinetics (3.9–8.6 mM; Morrison & James, 1965; Schimerlik & Cleland, 1973), suggesting active-site binding of this substrate. At concentrations of phosphocreatine ≥12.4 mM, the His-3, -4, and -6 resonances showed downfield shifts (Figure 3) due either to ancillary weaker binding sites for phospho-

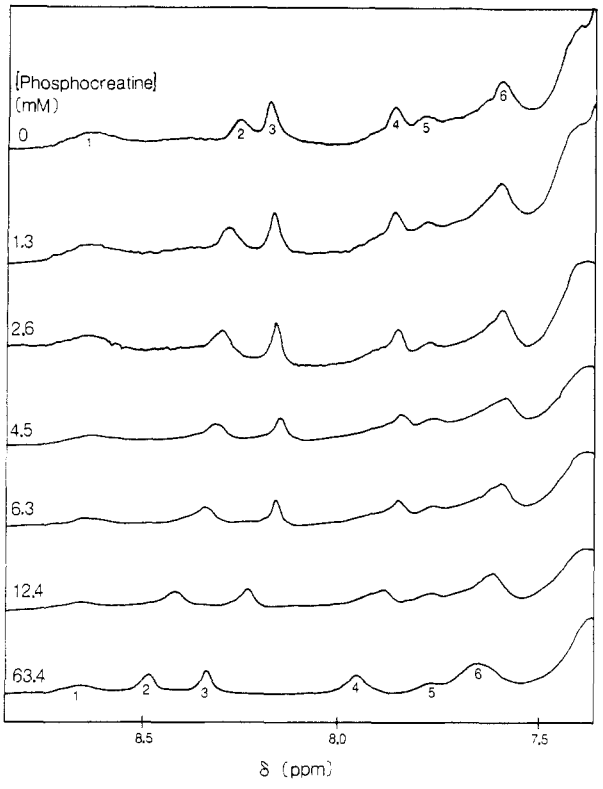

FIGURE 3: Effect of P-creatine on the histidine C-2 proton region of the 250-MHz proton NMR spectrum of creatine kinase. The sample contained 1.25 mM creatine kinase sites, 1 mM K^+Pipes buffer, pH* 6.8, in ^2H_2O in a total volume of 0.4 mL, and the indicated concentrations of phosphocreatine. NMR spectra were obtained at 250 MHz by using 1024 transients in four blocks of 256 transients each with 8192 data points, an acquisition time of 1.6 s, a spectral width of 3000 Hz, a line broadening of 2.0 Hz, quadrature phase detection, no delay time, 16-bit A/D conversion, a 75° pulse, and saturation by preirradiation of the H_2O signal. $T = 25$ °C. Histidine resonances correspond to His-1 through His-6 as in Figure 1.

Table VII: Dissociation Constants of Creatine Kinase-Substrate Complexes[a]

complex	K_D (mM) from Δ(δ) of His-2	K_D (mM) from Δ(δ) of His-6
E-(MgATP)	0.080 ± 0.006	0.088 ± 0.006
E-(MgADP)	*b*	0.11 ± 0.01
E-(P-creatine)	8.7 ± 0.1	*c*

[a] Determined by nonlinear least-squares computer fits to the titration data of Figure 4 assuming a 1:1 stoichiometry with creatine kinase sites. Components present were 1 mM K^+Pipes buffer, pH* 6.8. $T = 25$ °C. [b] Not determined due to broadening as a result of chemical exchange. [c] Chemical shift (δ) of resonance did not change as a function of substrate concentration.

creatine or to cooperativity in the binding of this substrate, manifested at these histidine sites. Titration with creatine failed to produce significant chemical shifts of the histidine resonances as expected from the negligible effects on the pK' values (Table VI, Figure 4A), with the exception of His-3 where only very small shifts were detected, preventing the determination of an accurate K_D.

MgATP caused progressive downfield shifts of the resonances of His-2 and -6 (Figure 5). The titration curves so obtained could be well fit (Figure 4) by using the parameters of Table VII. The K_D values of the MgATP used to fit the NMR titrations of His-2 (80 ± 6 μM) and His-6 (88 ± 6 μM) are in agreement with the K_D obtained by equilibrium dialysis (100 μM; Kuby & Noltmann, 1962) but are an order of magnitude lower than kinetically determined K_M values (0.7–3.2 mM; Morrison & James, 1965; Schimerlik & Cleland, 1973), suggesting a steady-state random kinetic scheme at pH 6.8 in the direction in which MgATP is the substrate. In the absence of MgATP, creatine may bind to the enzyme since it produces a small decrease in the pK' of His-3 (Table VI). If so, then either substrate can bind in absence of the other, which argues against a compulsory ordered mechanism.

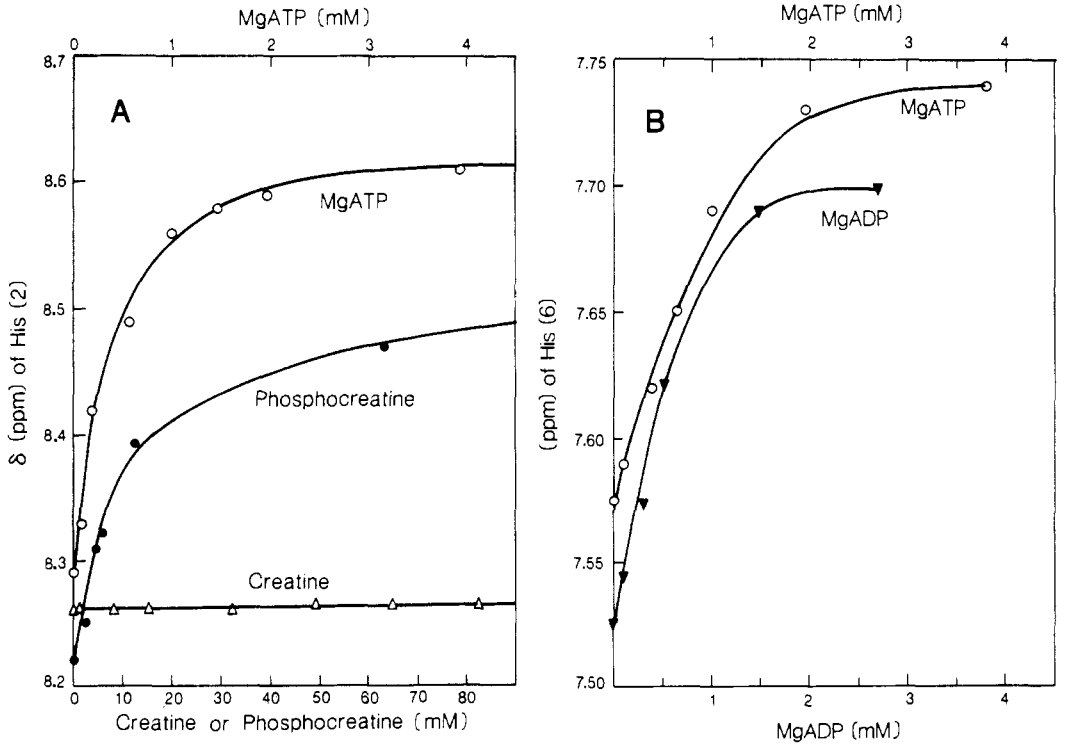

FIGURE 4: Titrations measuring the effects of substrates on the chemical shifts of the C-2 protons of His-2 (A) or His-6 (B) of creatine kinase. The curves are nonlinear least-squares computer fits to the data by using eq 2 with three variable parameters (K_D, and initial and final δ values) assuming a 1:1 stoichiometry between substrate and enzyme sites. The K_D values are given in Table VII. Conditions were otherwise as described in Figure 3.

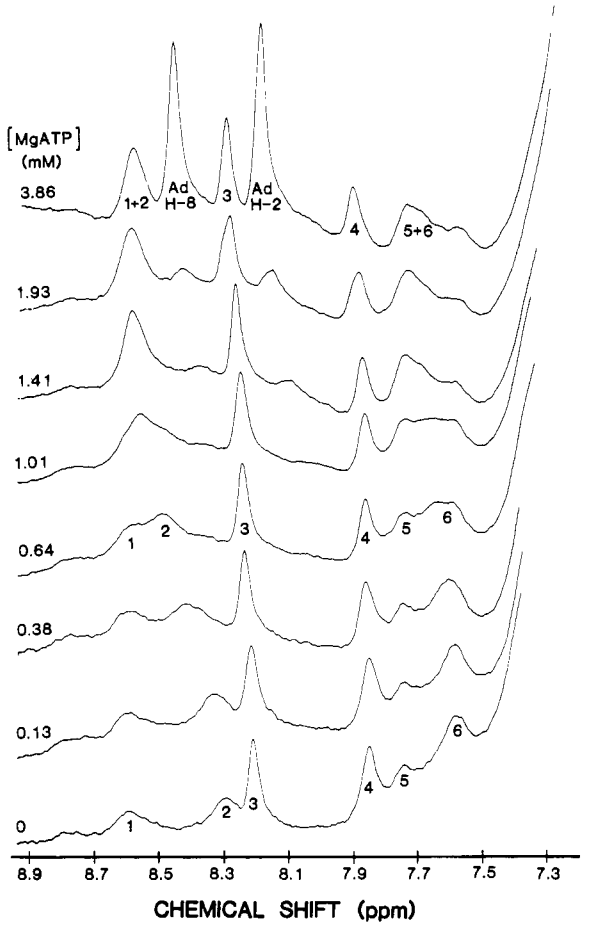

FIGURE 5: Effect of MgATP on the histidine C-2 proton region of the 250-MHz proton NMR spectrum of creatine kinase. The sample contained 0.83 mM creatine kinase sites, 1 mM K^+Pipes buffer, pH* 6.8, and the indicated concentrations of MgATP in ^2H_2O in a total volume of 0.4 mL. NMR spectra were obtained at 250 MHz by using 512 transients in two blocks of 256 transients each with 8192 data points, an acquisition time of 1.6 s, spectral width of 3000 Hz, a line broadening of 2.0 Hz, quadrature phase detection, no delay time, 16-bit A/D conversion, a 75° pulse, and saturation by preirradiation of the H_2O signal. T = 25 °C. Histidine resonances correspond to His-1 through His-6 as in Figure 1.

Titration of creatine kinase with MgADP caused a progressive downfield chemical shift of His-6. The dissociation constant of the enzyme–ADP·Mg^{2+} complex (0.11 ± 0.01 mM, Table VII) used to fit the titration curve of His-6 was in good agreement with the K_D (0.14–0.17 mM) obtained by kinetic analysis (Morrison & James, 1965; Schimerlik & Cleland, 1973), indicating active-site binding.

His-2 behaved in a more complicated manner in response to MgADP. The addition of a very small amount (0.09 equiv) of MgADP to creatine kinase at 25 °C resulted in a small (0.03 ppm) downfield shift and in an extreme broadening of the His-2 resonance such that it became difficult to observe. At a lower temperature (7 °C), 0.12 equiv of MgADP induced much less broadening. Raising the MgADP level at 7 °C continued to broaden this resonance and to shift it downfield until at a level of 0.69 equiv the resonance began to narrow. This behavior is consistent with exchange-limited transverse relaxation due to incomplete averaging of the chemical shifts of His-2 between those of the free enzyme and those of the enzyme-ADP·Mg^{2+} complex. The H-2 and H-8 protons of the adenine ring of MgADP also broadened during such titratons but did not significantly shift. Hence the kinetic parameters of the MgADP exchange could be obtained with greater accuracy from the $1/T_2$ value of the adenine protons.

An Arrhenius plot of the effect of five temperatures over the range 7–35 °C on $1/fT_{2D}$ of the adenine H-2 and H-8 protons was linear and consistent with exchange-limited relaxation, yielding by least-squares analysis an E_{act} of 8.7 ± 0.5 kcal/mol and a rate constant (k_{off}) for dissociation of the E–ADP·Mg^{2+} complex of 350 ± 50 s^{-1} at 25 °C. This k_{off} value for MgADP is 5-fold greater than k_{cat} of the creatine kinase reaction in the direction of MgADP formation (76 s^{-1}; Morrison & James, 1965), indicating that the E–ADP·Mg^{2+} complex detected by NMR dissociates rapidly enough to function in the creatine kinase reaction. The E_{act} and k_{off} values were used to calculate the absolute reaction rate parameters $\Delta H^{\ddagger}$ = 8.1 ± 0.5 kcal/mol, $-T\Delta S^{\ddagger}$ = 5.9 ± 0.5 kcal/mol (at 25 °C), and $\Delta G^{\ddagger}$ = 14.0 ± 0.7 kcal/mol (at 25 °C) for the dissociation of the E–ADP·Mg^{2+} complex. From k_{off} and the dissociation constant, the rate constant (k_{on}) for binding of MgADP to creatine kinase of (3.2 ± 0.3) × 10^6 M^{-1} s^{-1} was calculated. This value is significantly slower than would be expected for a diffusion-controlled process, providing further evidence for conformational changes coupled to the binding of MgADP. In addition to its line broadening of the His-2 resonance, MgADP at saturation induced a 10-Hz broadening of His-6. The substrates creatine, phosphocreatine, and MgATP also induced broadenings of His-6 by 5, 8, and 8 Hz, respectively, at saturation, due to changes either in the environment or in the mobility of this residue.

Effects of Cr^{3+}ATP on the Transverse Relaxation Rates of the Histidine Protons of Creatine Kinase. The effects of substrates on the pK' values (Table VII) and the chemical shifts (Figure 4) of the histidine resonances suggest that His-2 and His-6 are at the active site of creatine kinase, although indirect effects of protein conformational changes on these signals cannot be excluded. Proof of their presence at the catalytic site requires the detection of distance-dependent effects on the His-2 and His-6 resonances, such as paramagnetic effects on longitudinal and transverse relaxation rates.

The stable paramagnetic complex β,γ-bidentate Cr^{3+}ATP is a competitive inhibitor with respect to MgATP with a K_D of 68 ± 6 μM (Dunaway-Mariano & Cleland, 1980). The addition of 0.06 equiv of Cr^{3+}ATP to creatine kinase caused a selective broadening of the His-2 resonance and to a lesser extent of the His-6 resonance (Figure 6A,B). The subsequent addition of a saturating amount of phosphocreatine, which is known to compete with MgATP at the active site (Morrison & James, 1965), removed these broadenings (Figure 6C) and shifted the His-2 resonance downfield due to its effect on the pK' of His-2. In a related experiment (not shown), MgATP (1.14 mM) also reversed the effects of Cr^{3+}ATP (90.0 μM) on the line widths of the His-2 and His-6 resonances of creatine kinase (1.06 mM) and shifted the His-2 resonance downfield due to its effect on the pK'. These paramagnetic effects on $1/T_2$ by the substrate analogue Cr^{3+}ATP and their reversal by substrates indicate the presence of His-2 and His-6 at or near the catalytic site.

No measureable line broadening was detected of the C-2 resonance of His-3 or of the C-4 resonance, supporting its assignment as His-3′ (Figure 6).

A titration measuring the paramagnetic effects of Cr^{3+}ATP on the transverse relaxation rates of the histidine C-2 protons (Table VIII) reveals a maximum $1/fT_{2p}$ value of 517 ± 60 s^{-1} for the His-2 resonance, a somewhat smaller value for His-6, and negligibly small values for His-3 and His-4. The broadness of the His-1 and His-5 resonances precluded the measurement of their relaxation rates. The maximal $1/fT_{2p}$ value of His-2 sets a lower limit of ≥517 s^{-1} for k_{off} of Cr^{3+}-

Table VIII: Effect of $Cr^{3+}ATP$ on the Relaxation Rates of the Histidine Protons of Creatine Kinase at 250 MHz [a]

$Cr^{3+}ATP$ (μM)	His-2		His-3		His-4		His-6		His-3′ [c]
	$1/T_1$ (s^{-1})	$1/T_2$ (s^{-1}) [b]	$1/T_1$ (s^{-1})	$1/T_2$ (s^{-1}) [b]	$1/T_1$ (s^{-1})	$1/T_2$ (s^{-1}) [b]	$1/T_1$ (s^{-1})	$1/T_2$ (s^{-1}) [b]	$1/T_1$ (s^{-1})
0	1.76 ± 0.15	22	1.38 ± 0.10	26	1.89 ± 0.15	29	3.12 ± 0.15	34	2.16 ± 0.10
13.5	2.51 ± 0.25	28	1.11 ± 0.15	30	1.93 ± 0.20	29	3.72 ± 0.20	37	2.34 ± 0.20
35.5	3.05 ± 0.20	38	1.37 ± 0.15	30	1.87 ± 0.20	26	4.39 ± 0.30	36	2.76 ± 0.40
77.9	5.12 ± 0.25	63	1.81 ± 0.20	37	1.90 ± 0.15	31	6.10 ± 0.40	61	3.33 ± 0.50
98.4	5.85 ± 0.45	74	*d*	42	*d*	46	6.96 ± 0.50	69	3.88 ± 0.50
$1/fT_{1p}$ [e]	37.3 ± 5.0		≤7.0		≤3.5		34.6 ± 5.0		15.3 ± 1.7
$1/fT_{2p}$ [f]		517 ± 60		130 ± 60		≤60		312 ± 60	

[a] The solutions contained 1 mM K^+Pipes buffer in ^2H_2O, pH* 6.8, and 0.95 mM creatine kinase sites. $T = 25$ °C. [b] The errors in $1/T_2$ are ~6 s^{-1}. [c] Assigned as the C-4 proton of His-3. [d] Values were not determined. [e] The paramagnetic contribution to the longitudinal relaxation rates due to enzyme-bound $Cr^{3+}ATP$. $f = [Cr^{3+}ATP]_b/[\text{enzyme sites}]$. [f] The paramagnetic contribution to the transverse relaxation rates due to enzyme-bound $Cr^{3+}ATP$.

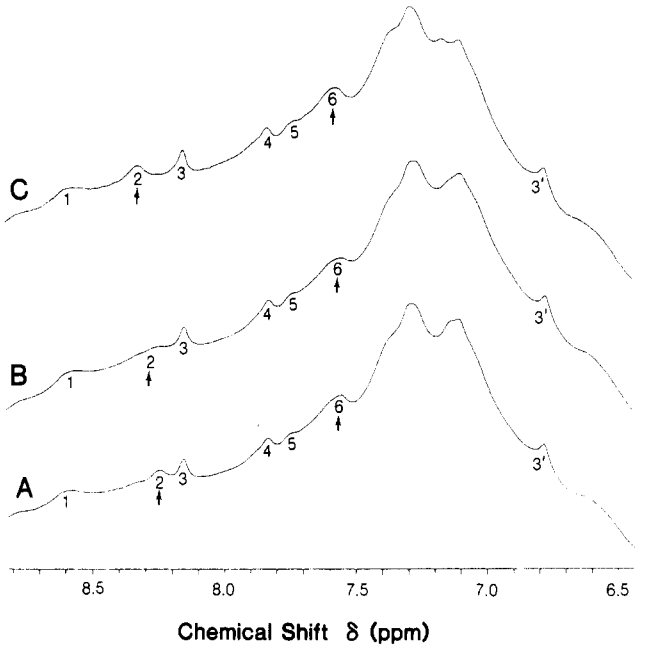

FIGURE 6: Paramagnetic effect of $Cr^{3+}ATP$ on the histidine resonances of creatine kinase as monitored by proton NMR at 250 MHz. (A) The sample contained 1.6 mM creatine kinase sites and 1 mM K^+Pipes buffer, pH* 6.8, in ^2H_2O in a total volume of 0.4 mL. (B) Components were as in (A) with 0.1 mM Cr^{3+} ATP. (C) Components were as in (B) with 5 mM P-creatine. NMR spectra were obtained at 250 MHz by using 2048 transients in eight blocks of 256 transients each of 8192 data points, an acquisition time of 1.6 s, spectral width of 3000 Hz, a linebroadening of 2.0 Hz, quadrature phase detection, no delay time, 16-bit A/D conversion, a 75° pulse, and saturation by preirradiation of of the H_2O signal. $T = 25$ °C.

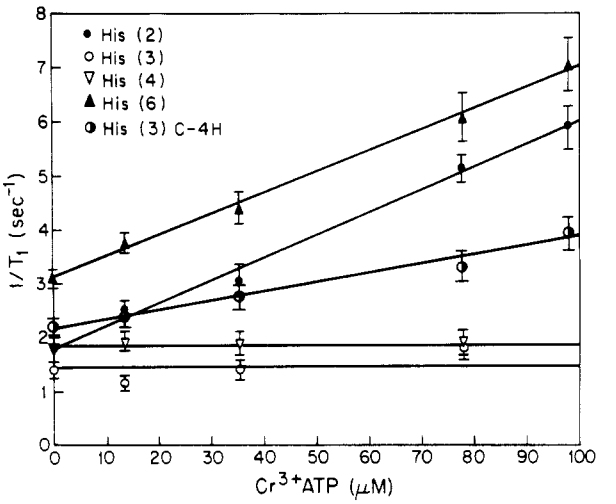

FIGURE 7: Paramagnetic effect of $Cr^{3+}ATP$ on the longitudinal relaxation rates ($1/T_1$) of the histidine proton resonances of creatine kinase. The symbols refer to the C-2 protons of His-2 (●), His-3 (○), His-4 (▽), His-6 (▲), and the C-4 proton assigned to His-3 (◑). The solid line is a computer fit to the $1/T_1$ data by using a linear least-squares analysis. Error bars on each point represent the average error of each measurement as obtained by computer analysis. NMR spectra of 0.95 mM creatine kinase sites were obtained at 250 MHz in 1 mM K^+Pipes buffer, pH* 6.8, by using 256 transients with 8192 data points, a line broadening of 2.0 Hz, spectral width of 3000 Hz, an acquisition time of 1.6 s, a delay time of 3 s, 16-bit A/D conversion, a 180°–τ–90° pulse sequence, and saturation by preirradiation of the H_2O signal. $T = 25$ °C.

ATP from the enzyme–ATPCr^{3+} complex. From this value and the dissociation constant of $Cr^{3+}ATP$ (68 μM; Dunaway-Mariano & Cleland, 1980), a lower limit for k_{on}, the rate constant for $Cr^{3+}ATP$ binding to creatine kinase $\geq(7.6 \pm 0.8) \times 10^6$ M^{-1} s^{-1}, is calculated.

Effects of $Cr^{3+}ATP$ on the Longitudinal Relaxation Rates of the Histidine Protons of Creatine Kinase. For determination of distances between the paramagnetic $Cr^{3+}ATP$ and the histidine residues, the longitudinal relaxation rates ($1/T_1$) of the His C-2 and C-4 protons were measured at 250 MHz in a titration with $Cr^{3+}ATP$ (Figure 7, Table VIII). Paramagnetic effects on the longitudinal relaxation rates of the C-2 protons of His-2 and His-6 and the C-4 proton of His-3 (3) were detected, and the $1/fT_{1p}$ values of these protons were calculated by linear least-squares analysis of the $Cr^{3+}ATP$ titration curves (Figure 7). No significant paramagnetic effects were detected on the $1/T_1$ values of the C-2 protons of His-3 and His-4 in accord with the $1/T_2$ studies (Table VIII).

When $1/fT_{1p}$ values are not limited by the rate of chemical exchange of $Cr^{3+}ATP$, they may be used in eq 4 and 5 to calculate Cr^{3+} to proton distance (r) (Mildvan & Gupta, 1978; Mildvan et al., 1980). In these equations, C is a constant

$$r = C[fT_{1p}[f(\tau_c)]]^{1/6} \quad (4)$$

$$f(\tau_c) = \frac{3\tau_c}{1 + \omega_I^2\tau_c^2} + \frac{7\tau_c}{1 + \omega_S^2\tau_c^2} \quad (5)$$

equal to 705 Å/$s^{1/3}$ for Cr^{3+}–proton interactions, ω_I and ω_S are the nuclear and electron precession frequencies, and τ_c is the dipolar correlation time. The exchange rate of $Cr^{3+}ATP$ ($\geq 517 \pm 60$ s^{-1}) estimated from the maximal $1/fT_{2p}$ value is an order of magnitude greater than the $1/fT_{1p}$ values (Table VIII), indicating that the latter are not exchange limited.

The correlation time was evaluated from the frequency dependences of $1/fT_{1p}$ for the His-2 and His-6 protons between 250 and 360 MHz (Table IX). For His-2 and His-6, the ratios ($1/fT_{1p}$ at 250 MHz)/($1/fT_{1p}$ at 360 MHz) were similar

Table IX: Calculation of Distances from Cr^{3+}ATP to Histidine Protons

His	$1/fT_{1p}$ at 250 MHz (s^{-1})	$(1/fT_{1p}$ at 250 MHz)/$(1/fT_{1p}$ at 360 MHz)	τ_c at 250 MHz ($s \times 10^{10}$)	$f(\tau_c)$ at 250 MHz ($s \times 10^{10}$)	r [c] (Å)
2	37.3	1.7 ± 0.2	8.71 [a], 4.36 [b]	9.10 [a], 8.91 [b]	12.0 ± 0.5
3	≤7.0				≥16 [d]
4	≤3.5				≥18 [d]
6	34.6	1.3 ± 0.2	3.96 [a], 3.32 [b]	8.57 [a], 7.83 [b]	12.0 ± 0.6
3'	15.3				13.8 ± 0.6 [d]

[a] τ_c assumed to be independent of ω_I. [b] τ_c assumed to be proportional to ω_I^2. [c] Errors in r take into account those in $1/fT_{1p}$ and in $f(\tau_c)$. [d] Average of τ_c for His-2 and His-6 used.

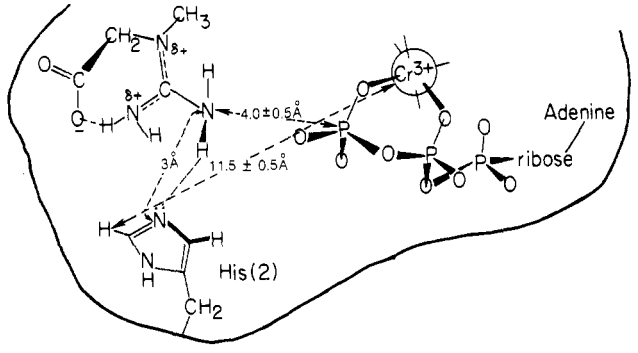

FIGURE 8: Hypothetical model of the creatine kinase–Cr^{3+}ATP complex showing the upper limit distance from Cr^{3+} to the C-2 proton of His-2. The model was built by assuming a reaction coordinate distance of 4.0 ± 0.5 Å (Mildvan, 1981) and an N–H---N hydrogen-bond distance of 3.0 Å (Donahue, 1968).

(1.5 ± 0.3) within experimental error (Table IX). These values were separately used as previously described (Mildvan & Gupta, 1978; Mildvan et al., 1980) to calculate the range of τ_c values at 250 MHz assuming no frequency dependence of τ_c and the maximal frequency dependence of τ_c (Bloembergen & Morgan, 1961). The resulting range of τ_c values (3.3×10^{-10} to 8.7×10^{-10} s, Table IX) is typical for the electron spin relaxation time of enzyme-bound Cr^{3+}–nucleotide complexes (Cleland & Mildvan, 1979). This range yielded, from eq 5, a much narrower range of $f(\tau_c)$ values (7.8×10^{-10}–9.1×10^{-10} s, Table IX). From the $f(\tau_c)$ and the $1/fT_{1p}$ values, distances from Cr^{3+}ATP to the protons of His-2, -3, and -6 were calculated with eq 4 (Table IX). The errors in the distances take into account the errors in $1/fT_{1p}$ (Table VIII) and in $f(\tau_c)$ (Table IX). The distances, which range from 12 to 14 Å, establish the proximity of the C-2 protons of His-2 and His-6 and the C-4 proton of His-3 to the metal–nucleotide substrate binding site of creatine kinase and are consistent with the role of His-2 as the general acid/base catalyst. Although the 12.0 ± 0.6 Å distance from the Cr^{3+} of enzyme-bound Cr^{3+}ATP to the C-2 proton of His-2 seems rather large, model building studies summarized in Figure 8 yield an upper limit of 11.5 ± 0.5 Å for such a distance to the C-2 proton of an imidazole which is accepting a hydrogen bond from a guanidinium NH_2 of creatine, which in turn is 4.0 ± 0.5 Å from the γ-P of Cr^{3+}ATP. The latter value is a reasonable reaction coordinate distance for an associative mechanism (Mildvan, 1981) and is consistent with the existence of transition state complexes (Watts, 1973) and with a recent measurement of the Cr^{3+} to phosphorus distance of 6 Å in the creatine kinase–ADPCr^{3+}–phosphocreatine complex (Gupta, 1980). Hence His-2 could interact with creatine as the general acid/base catalyst, even though it is 12.0 ± 0.6 Å from the metal activator. This distance could decrease as the transition state is approached.

His-6, which is located at a comparable distance from the Cr^{3+}, is an unlikely candidate for the general acid/base catalyst because of its low pK' value compared to that detected by kinetic studies (Cook et al., 1981). The increase in the pK' value of His-6 in response to the binding of phosphorylated substrates may reflect a direct electrostatic effect or, less likely, indirect conformational effects of substrate binding.

While no significant paramagnetic effects of Cr^{3+}ATP on $1/T_1$ of the C-2 proton of His-3 and His-4 were detected, the errors in the data (Figure 7, Table VIII) were used to estimate upper limit $1/fT_{1p}$ values and lower limit distances to these protons of ≥16 Å and ≥18 Å, respectively (Table IX). The lower limit distance from Cr^{3+} to the C-2 proton of His-3 (≥16 Å) is consistent with the measured Cr^{3+} to C-4 proton distance of 14 Å (Table IX) since these distances could differ from each other by 0–4.3 Å, depending on the orientation of the imidazole ring. The large distance of His-4 from Cr^{3+}ATP (≥18 Å) suggests that the increases in its pK' values in response to the binding of phosphorylated substrates result from indirect conformational effects.

Conclusions

Three histidine residues have been found to be at or near the active site of creatine kinase. One of these, His-2, has a pK' value in the free enzyme of 7.0 which increases by 0.6–0.7 unit when phosphorylated substrates are bound, in quantitative agreement with the results of a pH–rate study of this enzyme (Cook et al., 1981), indicating His-2 to be the general acid/base catalyst which deprotonates the guanidinium group of creatine as it is phosphorylated by Mg ATP. The 12.0 ± 0.6 Å distance of the His-2 C-2 proton from the paramagnetic Cr^{3+} of enzyme-bound Cr^{3+} ATP is consistent with this role. His-6, which is also 12 Å from the Cr^{3+}, may interact electrostatically with the phosphorylated substrates, as suggested by the increase in its pK' when these substrates are bound. His-3 is somewhat farther from Cr^{3+} ATP and is so positioned that its C-4 proton is oriented toward the Cr^{3+} since no significant paramagnetic effect was detected on $1/T_1$ of its C-2 proton. Because the pK' value of His-3 is not greatly altered by the binding of substrates, no catalytic role for this residue has been demonstrated. It may be near the adenine ring where histidine residues have recently been detected by NOE studies of pyruvate kinase and adenylate kinase (Meshitsuka et al., 1981b). Although the phosphorylated substrates phosphocreatine and MgATP produced small but significant increases in the pK' of His-4, these probably represent remote conformational effects since this residue is far (≥18 Å) from the substrate binding site.

Acknowledgments

We are grateful to Dr. G. M. Smith and Dr. G. H. Reed for useful discussions throughout the course of this work.

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Unfolding of *lac* Repressor and Its Proteolytic Fragments by Urea: Headpieces Stabilize the Core within *lac* Repressor†

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ABSTRACT: Circular dichroism measurements were used to compare the urea-induced unfolding transition of the *lac* repressor with those of its separated tryptic fragments, the tetrameric core, and the N-terminal headpiece. The presence of the headpieces covalently linked to the core in the intact repressor leads to a stabilization against urea denaturation as compared to that for the isolated core. This results in a shift of the midpoint of the transition by about 0.5 M urea. When the inducer isopropyl β-D-thiogalactoside is bound, the core is stabilized more than the entire repressor. The isolated headpiece is considerably more stable against urea denaturation than the tryptic core or the *lac* repressor. The reversible denaturation process of the headpiece was quantitatively analyzed, and the free energy of unfolding in the absence of urea was found to be 2.4 or 2.9 kcal/mol, depending on the method of calculation used. Comparison between the circular dichroism spectra of the *lac* repressor, the tryptic core of the *lac* repressor, and the headpiece supply further evidence that there are no major conformational differences between the structural domains (core and headpieces) before and after proteolytic cleavage of the *lac* repressor. These results are discussed with respect to the contacts between the different domains of the protein. It is concluded that relatively weak interdomain contacts are probably responsible for the stabilization of the core by the covalently linked headpieces and that these contacts might be weakened upon binding of the inducer.

The *lac* repressor is a tetrameric protein which controls the expression of the structural genes of the *lac* operon by binding to the *lac* operator (Müller-Hill, 1975; Bourgeois & Pfahl, 1976). There is no doubt now that this protein, like many other proteins, is composed of several domains. These domains, which may be obtained by limited proteolysis of the *lac* repressor at high ionic strength, are a tetrameric core (4 × residues 60–360) and four headpieces (residues 1–51 or 1–59, depending on the time of the hydrolysis). The tetrameric core

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0006-2960/81/0420-6164$01.25/0