NMR Assignments of the Four Histidines of Staphylococcal Nuclease in Native and Denatured States†

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Received August 11, 1987; Revised Manuscript Received November 17, 1987

ABSTRACT: NMR signals from all four histidine ring C_ϵ protons and three of the four histidine C_δ protons in the protein staphylococcal nuclease have been assigned by comparing spectra of the wild-type (Foggi strain) protein to spectra of three variants that each lack a different histidine residue. All proteins studied were cloned and overproduced in *Escherichia coli*. The NMR spectra of the three mutant proteins (H8R, H46Y, and H124L) used to make these assignments were similar to one another and to those of the wild type, except for signals from the mutated residues. The pK_a values of those histidines conserved between the wild type and the mutants remained essentially unchanged. Multiple histidine C_ϵ proton resonances due to nonnative forms of nuclease were observed in both thermally induced and acid-induced unfolding. Residue-specific assignments of H_ϵ protons in the thermally denatured forms of the mutant H46Y were obtained from connectivities to the native state by saturation transfer.

Staphylococcal nuclease is a 16 807-dalton protein of 149 residues. It has no disulfide bonds. Its crystal structure has been solved to 1.5-Å resolution (Cotton et al., 1979). The protein belongs to the $\alpha + \beta$ class of proteins. It consists of five antiparallel β-pleated sheets arranged in a β-barrel and surrounded by three α-helices.

Staphylococcal nuclease is produced naturally by *Staphylococcus aureus*, where its metabolic function remains unclear. To facilitate purification of large amounts of native and mutant protein, the nuclease gene from *S. aureus* (Foggi strain) has been cloned into *Escherichia coli*, in a construct that causes overproduction of the protein when the level of phosphate in the growth media is kept low (Shortle, 1986).

Over 80 point mutations in this gene have been obtained by shotgun mutagenesis (Shortle & Lin, 1985), and virtually any desired point mutation can be constructed by using standard M13 oligonucleotide directed mutagenesis (Bauer et al., 1985; Kunkel, 1985). This makes the nuclease system amenable to studies of the effects of point mutations on protein folding, structure, and function (Shortle & Meeker, 1986; Hilber et al., 1986; Serpersu et al., 1987).

Numerous reports have been published on the folding/unfolding of nuclease [early work is reviewed by Tucker et al. (1979)]. Evidence from NMR[1] spectroscopy has accumulated that under some conditions the unfolding of nuclease does not obey the classical two-state model of denaturation: (1) Early NMR studies of the aromatic region of nuclease at 100 MHz (Markley et al., 1970) indicated that His^{46} exists in two slowly exchanging environments at low pH. (2) A 220-MHz study of the acid-induced denaturation of nuclease at room temperature (Epstein et al., 1971) showed that the pH midpoint of denaturation, measured from the intensity of H_ϵ^{121}, was slightly higher ($pK_f = 4.1$) than the midpoint measured from the other three H_ϵ resonances ($pK_f = 3.9$). (3) Recently, a 500-MHz study (Fox et al., 1986) revealed multiple peaks for all four H_ϵ resonances at pH 5.5. The smaller peaks were shown by saturation transfer to be due to a second form of nuclease in slow exchange with the major native form.

The common feature of all these experiments was that the four histidines of nuclease (at position 8, 46, 121, and 124) were used as reporter groups to monitor changes in the molecule. The H_ϵ resonances are by far the best resolved resonances in the spectrum. The H_ϵ resonances of nuclease were assigned tentatively by Markley (1969), but the assignments were criticized for not being sufficiently rigorous (Anfinsen & Scheraga, 1975). The H_ϵ^{124} assignment was based on comparisons of spectra of nuclease from the Foggi strain (referred to as wild type here) and the V8 strain, which differs from wild type only by having a Leu substituted for His^{124}. The assignment of H_ϵ^{46}, which is close to the active site, was based on changes in chemical shift when Ca^{2+} and the inhibitor pdTp were added (Markley, 1969; Cozzone & Kaptein, 1983). The H_ϵ^8 and H_ϵ^{121} assignments were the weakest ones, since they were based on the argument that His^8, which appears more disordered and exposed in the crystal structure than His^{121}, should give rise to NMR chemical shifts and a pK_a value closer to that of model histidine compounds. Recently, Serpersu and Mildvan (1987) presented evidence that the assignments of H_ϵ^8 and H_ϵ^{121} should be reversed. They found that the assignments of Markley (1969) were inconsistent with their T_1 data for a Mn^{2+} complex of nuclease, assuming that Mn^{2+} binds in the Ca^{2+} site identified by X-ray crystallography.

† Supported by NIH Grant GM-35976 and NSF Grant DMB-84-10222. This study made use of the National Magnetic Resonance Facility at Madison, which is supported by NIH Grants RR-023021 and RR-02781, the University of Wisconsin, and the U.S. Department of Agriculture.

[1] Abbreviations: NMR, nuclear magnetic resonance; ppm, parts per million; TSP, 3-(trimethylsilyl)propionate-d_4; pK_f, pK for folding; His, histidine; Tyr, tyrosine; H_ϵ, resonance from the histidine C_ϵ proton; H_δ, resonance from the histidine C_δ proton; H8R, nuclease with His^8 changed to Arg^8; H46Y, nuclease with His^{46} changed to Tyr^{46}; H124L, nuclease with His^{124} changed to Leu^{124}; NOE, nuclear Overhauser effect; pdTp, thymidine 3′,5′-bisphosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SDS, sodium dodecyl sulfate; DMSO, dimethyl sulfoxide; COSY, correlated spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; HOHAHA, homonuclear Hartmann–Hahn spectroscopy.

0006-2960/88/0427-2158$01.50/0

Because of the revived interest in the four H_ϵ resonances of nuclease, we decided to use modern protein engineering techniques in an attempt to achieve a more rigorous assignment of these four resonances. The new assignments for the H_ϵ resonances of nuclease are in complete agreement with those of Markley (1969).

Experimental Procedures

Materials. The bacterial strain *E. coli* SE6004 and the plasmids for the wild type (pFOG405), H46Y (B204), and H124L (Z101) were gifts of Dr. D. Shortle (Johns Hopkins University). The bacterial strain *E. coli* JM105 and the phage strains M13mp11am and M13mp11wt were supplied by Dr. J. Gardiner (University of Illinois—Urbana). The bacterial strain *E. coli* RZ1032 was constructed from the *E. coli* strain BW313 HfrKL16 Po/45 [LysA(61–62)], thi-1, rel A1, spoT1, dut-1, ung-1, and was a gift of Dr. W. Reznikoff (University of Wisconsin—Madison). This strain supported growth of phage-containing amber mutants and produced phage containing uracil in the DNA.

Creation of the H8R Mutant. The approach used to make H8R was a combination of the oligonucleotide-directed mutagenesis methods of Kunkel (1985) and Bauer et al. (1985). The nuclease gene was excised from the pFOG405 expression vector (Shortle, 1986) by using the enzymes *Eco*RI (BRL) and *Sal*I (New England Biolabs) and inserted into an M13 vector (M13mp11am) containing amber (am) mutations within phage genes I and II. This vector cannot grow in a suppressor-free bacterial strain. The vector then was grown in an *E. coli* dut$^-$ ung$^-$ strain (RZ1032), thus incorporating uracils into the vector genome (Kunkel, 1985). A gapped duplex was formed by annealing portions of a complementary (−) strand containing wild-type copies of genes I and II to the am-uracil-containing (+) strand. The mutagenic oligonucleotide was then annealed, and the remaining gaps were filled and sealed enzymatically. Next, the covalently closed DNA was transformed into *E. coli* strain JM105 (sup^0). The choice of a host that was suppressor free and dut$^+$ ung$^+$ enabled the rigorous enrichment of the complementary (−) mutagenic strand. The mutant was identified further by differential hybridization (Wallace et al., 1981) and confirmed by dideoxy sequenching (Sanger et al., 1977). The gene for overexpression of nuclease was subsequently excised from M13 and subcloned into PBR322. This construct (pFOG405-H8R) was transformed into *E. coli* strain SE6004 and used to express nuclease.

Purification of Proteins. Cells were grown in MOPS (Research Organics) media in either 10- or 35-L fermentors. The protein was purified according to the method of Shortle (1986). Typical yields were 2–4 mg of nuclease/L of culture.

Preparation of NMR Samples. Purified nuclease was dialyzed once against 50 mM phosphate, pH 7, and 0.1 M NaCl; once against 10 mM phosphate, pH 7, and 0.1 M NaCl; and 3 times against deionized, distilled water. During the last procedure a white precipitate often formed. The precipitate was removed by centrifugation for 10 min at 10000 rpm. The supernatant was lyophilized, taken up in a 0.3 M NaCl solution in 98% D_2O (Aldrich), and lyophilized twice from 98% D_2O and 2 times from 100% D_2O (Aldrich). The sample was then taken up in a volume of 100% D_2O such that the sample was 0.3 M in NaCl. The purity of all samples was checked by SDS–polyacrylamide gel electrophoresis. All samples were >90% pure.

^{1}H NMR Spectroscopy. All the data for this report were collected at 500 MHz on a Brucker AM-500 spectrometer and processed by using an ASPECT-3000 computer. TSP was used as an internal standard. Except where noted, a PRESAT pulse sequence with a D1 delay of 2 s was used to suppress the residual water peak. Free induction decays (FIDs) were obtained by using quadrature detection and digitized into 8K data point arrays.

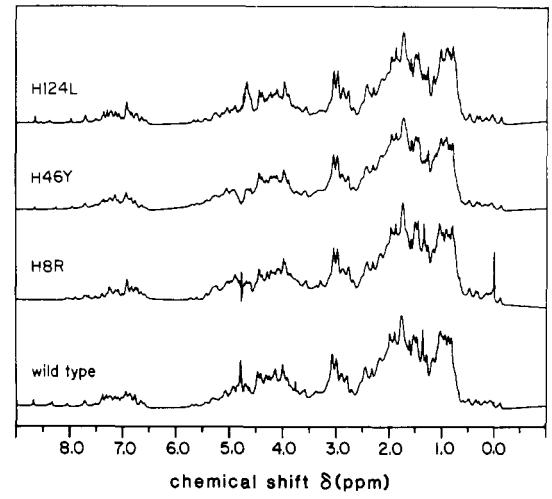

FIGURE 1: ^{1}H NMR spectra (500 MHz) of wild-type staphylococcal nuclease and the three mutants used to assign the four histidines. The figure illustrates the great similarity observed between the spectra of wild-type nuclease and the mutant forms. Conditions: (wild type) 5.2 mM, pH 5.5, 16 transients; (H8R) 0.52 mM, pH 6.45, 512 transients; (H46Y) 3.3 mM, pH 5.7, 128 transients; (H124L) 2 mM, pH 5.5, 256 transients. All protein samples were 0.3 M in NaCl, and all of the spectra were collected at 298 K.

pH Titrations. The pH was adjusted by addition of ^{2}HCl or KO^{2}H. All pH measurements were made at room temperature. The pH was measured both immediately before and immediately after obtaining NMR spectra. The values reported here are the average of the two measurements. Differences between the two measurements typically were 0.02 pH units. It should be noted that all pH and pK_a values reported here are pH* and pK_a*; that is, they were not corrected for the deuterium isotope effect. The acid-induced denaturation was completely reversible, and no precipitation occurred.

Thermal Denaturation. The H46Y sample that was used for the temperature titration and for the assignment of H_ϵ resonances in the denatured state was heated to 315 K, at pH 5.7, for a few minutes before use. This procedure exchanged out all amide protons in H46Y not removed during lyophilization. During the temperature titration, care was taken to heat the sample slowly. The temperature was raised in 1-deg increments, and the sample was allowed to equilibrate at a given temperature for 15 min before collecting spectra. The temperatures obtained from the heating unit were calibrated to an 80% ethylene glycol/DMSO standard. Denaturation in the temperature range used in this study was completely reversible, as monitored by the return of resonances characteristic of the native state which appeared when the sample was cooled. No precipitation was seen during the titration.

Results and Discussion

Assignment of Histidine Resonances. NMR is a powerful technique for studying protein structure because it can selectively monitor the environment of individual nuclei in the molecule. In order to interpret NMR data in structural terms, however, it is necessary to assign the resonances in the NMR spectrum to the specific nuclei that give rise to them. It is often easy to identify a resonance with a residue type, but it is considerably more difficult to make assignments to specific

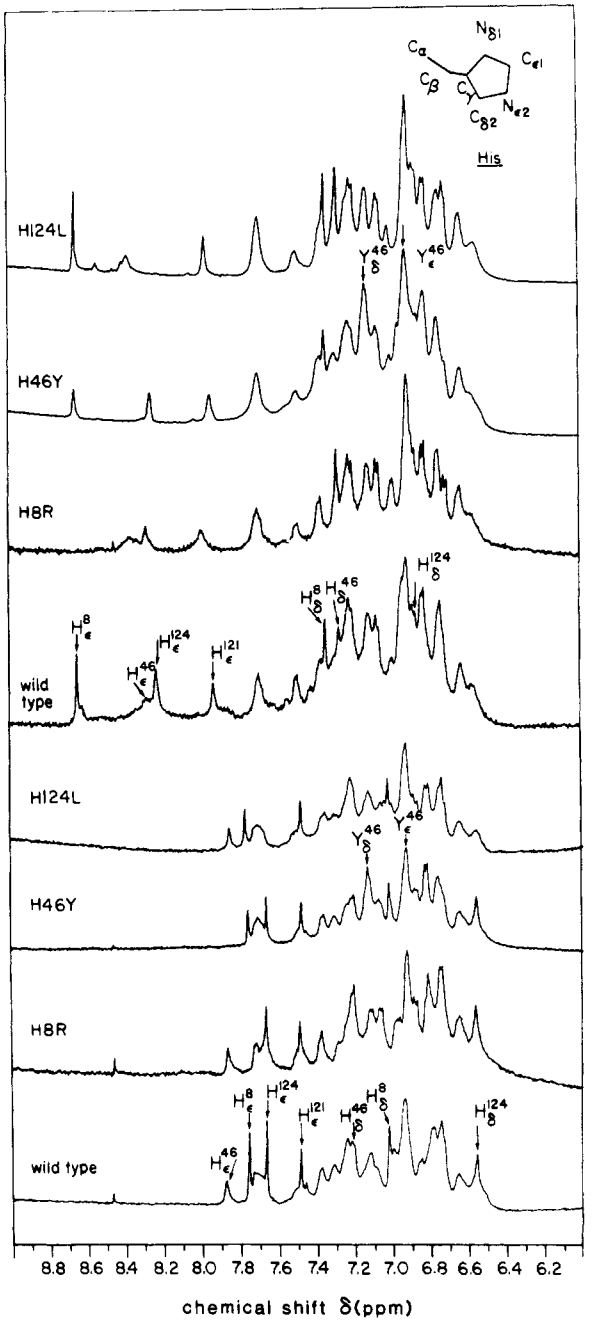

FIGURE 2: Spectra of wild-type nuclease and mutants H8R, H46Y, and H124L at acidic and basic pH. Top four spectra: (H124L) 2 mM, pH 5.5, 256 transients; (H46Y) 3.3 mM, pH 5.7, 128 transients; (H8R) 0.53 mM, pH 5.65, 512 transients; (wild type) 0.9 mM, pH 5.77, 128 transients. Bottom four spectra: (H124L) 2 mM, pH 8.35, 264 transients; (H46Y) 1.85 mM, pH 8.55, 256 transients; (H8R) 0.45 mM, pH 8.48, 512 transients; (wild type) 2.5 mM, pH 8.5, 128 transients. All solutions contained 0.3 M NaCl; all spectra were collected at 298 K. The inset to this figure shows the labeling scheme for the heavy atoms in histidine. Assignments for both H_ϵ and H_δ protons are indicated in the spectrum of wild type. Assignments for the aromatic protons of Tyr^{46}, which is the substituted residue in H46Y, are indicated in the spectrum of H46Y (see also Figure 4). The resonance near 8.5 ppm in the spectra of some samples is due to a formate impurity.

amino acids in the sequence. Two-dimensional NMR techniques such as COSY (Wüthrich et al., 1982), NOESY (Wagner & Wüthrich, 1982), and HOHAHA (Davis & Bax, 1985) are now available for making sequential assignments for almost all the amino acids in a small protein, but these methods are difficult to apply universally, especially for proteins with molecular weights above 10 000 such as nuclease.

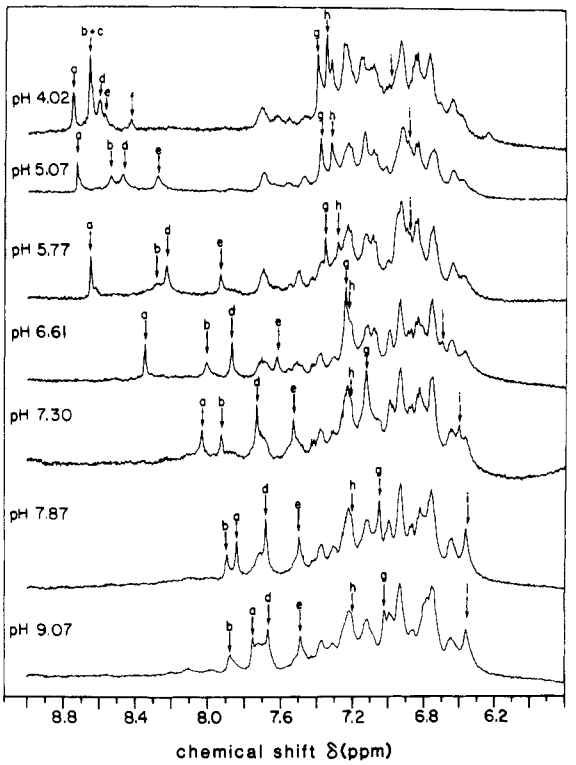

FIGURE 3: Selected spectra from the pH titration of wild-type nuclease. Conditions: 0.9 mM, 0.3 M NaCl; all spectra acquired at room temperature. Each spectrum resulted from the sum of 128 accumulations. Legend: a, H_ϵ^8; b, H_ϵ^{46}; c, denatured H_ϵ; d, H_ϵ^{124}; e, H_ϵ^{121}; f, $H_\epsilon^{121'}$; g, H_δ^8; h, H_δ^{46}; i, H_δ^{124}.

For proteins as large as nuclease new strategies need to be applied to the assignment problem. One approach is to compare spectra of the wild type to mutants that differ by single amino acid substitutions (Markley et al., 1986). A critical consideration when this method is used is that the mutation does not affect the structure and, consequently, the spectral characteristics of residues not involved. This criterion appears to be met for all the mutants we will consider in this paper.

The histidine residues we chose to mutate, located at positions 8, 46, and 124, are all on the surface of the molecule, and their side chains do not contribute significantly to the structure. Figure 1 compares the spectra of wild-type nuclease and the three mutants H8R, H46Y, and H124L. The spectra are all quite similar. Additional evidence that the structures of the mutants are similar to that of the wild type is provided by similarities in the pK_a values of the conserved histidines (Table I), the enzymatic activities, and the denaturation behavior of all the proteins.

Histidine residues have a special place in ^1H NMR because of their unique spectral properties. The inset of Figure 2 shows a histidine residue with the nomenclature used in this report. Most often in proteins the N_ϵ site is occupied, and protonation occurs at N_δ as the pH is lowered. The C_ϵ proton (H_ϵ) resonances of histidines are often the most downfield-shifted resonances in proteins, and their chemical shifts are very sensitive to pH. The chemical shift changes between acidic and basic end points average 1 ppm, with the midpoint usually falling between pH 4 and pH 8. The top four spectra in Figure 2 show the aromatic region of the nuclease spectrum at acidic pH, while the lower four spectra in Figure 2 show the same region at basic pH. Figure 3 shows selected pH points from the titration of wild type. The histidine assignments are indicated in these figures. Three assignments (H_ϵ^8, H_ϵ^{46}, H_ϵ^{124}) were obtained by comparing spectra of mutants to those of

Table I: pK_a Values for Nuclease Histidines[a]

resonance	wild type	H8R	H46Y	H124L
E_ϵ^8				
pK_a	6.82 ± 0.01		6.78 ± 0.02	6.78 ± 0.01
low-pH plateau	8.752 ± 0.003		8.76 ± 0.01	8.74 ± 0.01
high-pH plateau	7.73 ± 0.01		7.73 ± 0.01	7.74 ± 0.01
Hill coeff	0.88 ± 0.02		0.88 ± 0.02	0.95 ± 0.03
H_ϵ^{46}				
pK_a	5.80 ± 0.01	5.86 ± 0.02		5.90 ± 0.02
low-pH plateau	8.68 ± 0.01	8.67 ± 0.01		8.68 ± 0.01
high-pH plateau	7.880 ± 0.003	7.78 ± 0.06		7.855 ± 0.003
Hill coeff	0.90 ± 0.02	0.90 ± 0.03		0.80 ± 0.02
H_ϵ^{124}				
pK_a	5.99 ± 0.02	5.91 ± 0.02	5.88 ± 0.02	
low-pH plateau	8.61 ± 0.01	8.61 ± 0.01	8.64 ± 0.01	
high-pH plateau	7.65 ± 0.01	7.67 ± 0.01	7.66 ± 0.01	
Hill coeff	0.85 ± 0.03	0.94 ± 0.03	0.81 ± 0.03	
H_ϵ^{121}				
pK_a	5.49 ± 0.03	5.51 ± 0.02	5.42 ± 0.03	5.38 ± 0.02
low-pH plateau	8.66 ± 0.02	8.62 ± 0.01	8.68 ± 0.02	8.71 ± 0.02
high-pH plateau	7.48 ± 0.01	7.49 ± 0.01	7.48 ± 0.01	7.482 ± 0.004
Hill coeff	0.73 ± 0.02	0.84 ± 0.02	0.77 ± 0.04	0.81 ± 0.02
$H_\epsilon^{121\prime}$[b]				
pK_a	5.72 ± 0.02	ND	5.62 ± 0.03	5.50 ± 0.02
low-pH plateau	8.45 ± 0.01	ND	8.48 ± 0.01	8.60 ± 0.01
high-pH plateau	7.49 ± 0.01	ND	7.48 ± 0.01	7.485 ± 0.004
Hill coeff	0.90 ± 0.03	ND	0.86 ± 0.05	0.88 ± 0.02
H_δ^8				
pK_a	6.82 ± 0.02		6.76 ± 0.03	6.85 ± 0.03
low-pH plateau	7.392 ± 0.002		7.396 ± 0.004	7.384 ± 0.004
high-pH plateau	7.012 ± 0.004		7.01 ± 0.01	7.016 ± 0.004
Hill coeff	0.87 ± 0.03		0.89 ± 0.06	0.93 ± 0.06
H_δ^{46}				
pK_a	5.74 ± 0.05	5.94 ± 0.09		5.87 ± 0.07
low-pH plateau	7.336 ± 0.003	7.34 ± 0.01		7.34 ± 0.01
high-pH plateau	7.203 ± 0.003	7.199 ± 0.004		7.206 ± 0.003
Hill coeff	1.50 ± 0.20	0.76 ± 0.10		0.91 ± 0.12
H_δ^{124}				
pK_a	6.25 ± 0.09	5.96 ± 0.09	5.86 ± 0.11	
low-pH plateau	7.00 ± 0.02	7.00 ± 0.02	6.98 ± 0.02	
high-pH plateau	6.54 ± 0.02	6.56 ± 0.01	6.54 ± 0.01	
Hill coeff	0.67 ± 0.10	0.70 ± 0.08	0.52 ± 0.06	

[a] Conditions: (wild type) 0.9 mM, no. of pH points for titration = 21; (H8R) 0.45 mM, no. of points = 18; (H46Y) 1.85 mM, no. of points = 22; (H124L) 2 mM, no. of points = 31. All samples were 0.3 M in salt; all spectra were taken at 298 K. The values given in this table were calculated from plots of chemical shift vs pH by using a nonlinear least-squares regression program. Because the native and acid-denatured states of nuclease are in slow exchange, samples in the low-pH region give results for both states. The errors reported in this table are standard deviations from the least-squares regression and do not take into account the errors derived from pH measurements. We feel that the pK_a values reported are only good to ±0.1 pH units. [b] Since the minor resonance from H_ϵ^{121} only appears below pH 4.8, the values given for peak $H_\epsilon^{121\prime}$ were calculated by using the data from peak H_ϵ^{121} in the pH region 10–4.8 and the chemical shift of $H_\epsilon^{121\prime}$ below pH 4.8. In other words, to compute the pK_a for $H_\epsilon^{121\prime}$, we used the data for H_ϵ^{121}, except the low-pH plateau due to $H_\epsilon^{121\prime}$.

wild type. The chemical shift of the resonance assigned as H_ϵ^{121} is strongly dependent on pH, which allows us to identify it as histidine; its residue-specific assignment to His^{121} follows by induction.

In addition to assigning the histidine residues in nuclease, we were able to assign the aromatic resonances of Tyr^{46}, the substituted tyrosine in H46Y. These resonances occur in a more crowded region of the spectrum than the H_ϵ resonances and are consequently more difficult to resolve. To obtain an assignment, we needed to compare double-quantum-filtered COSY spectra of wild type and H46Y and to generate a difference spectrum (wild type − H46Y) (see Figure 4).

Figure 5a is a plot of the H_ϵ resonances of wild-type nuclease as a function of pH. Note that the H_ϵ^8 and H_ϵ^{46} resonances cross over at pH 7.66. Figure 5b shows the same type of plot for the H_δ resonances of histidines 8, 46, and 124. The assignments for the H_δ resonances were obtained from both a comparison of the spectra of wild type and the mutants and a comparison between the pK_a values of the H_ϵ and H_δ protons (see Figures 2 and 3 and Table I). Since both parameters reflect the pK_a values for the same residue, it follows that pK_a values obtained from H_ϵ and H_δ resonances must be the same. Matching up the pK_a values of H_δ peaks to those obtained for H_ϵ peaks allows assignments of the H_δ peaks. We were unable to assign H_δ^{121}. This resonance should occur between 6.6 and 7.4 ppm, but because of the poor resolution in the region we were unable to pick it out.

Thermal Denaturation of H46Y. It has been known for some time that a minor form of nuclease appears at low pH. Nondenaturing gels of nuclease purified by crystallization at pH 4.3 revealed two bands (Taniuchi & Anfinsen, 1966). On further purification by preparative electrophoresis, each band showed two equivalent bands when rerun at pH 4.3. The first NMR evidence for such a minor form came from observed splitting of the H_ϵ^{46} resonance into two peaks at low pH (Markley et al., 1970). Recently, Fox et al. (1986) showed that, at pH 5.5, minor peaks for all four H_ϵ resonances of nuclease are present. Saturation transfer revealed that the minor form responsible for these resonances is in slow exchange with both the native and denatured states. We have observed these minor resonances in all the mutants discussed in this paper. Figure 6 shows the changes as a function of temperature in the mutant H46Y. Note in the spectrum at 315 K the presence of two minor resonances for H_ϵ^8; this implies the

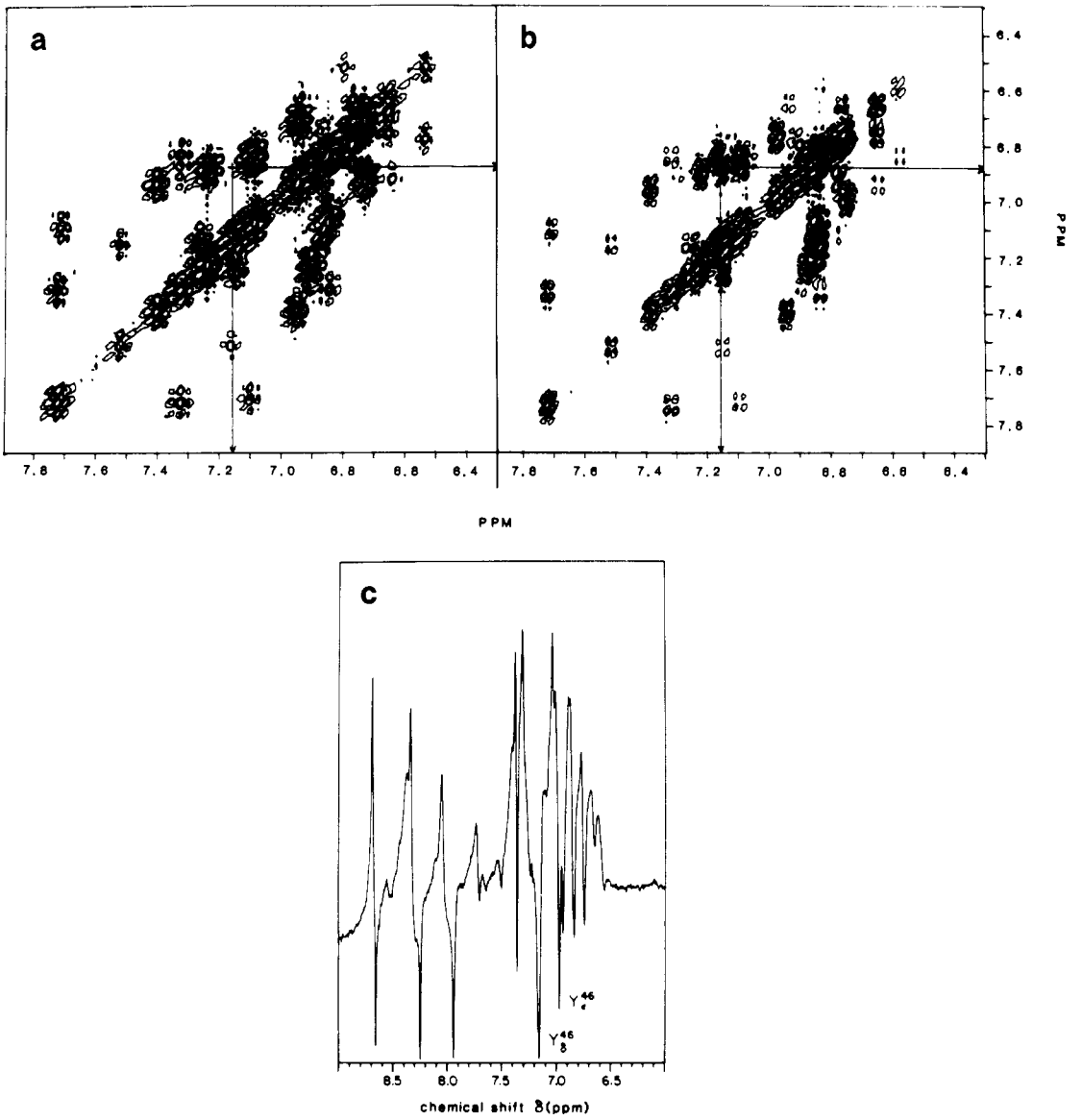

FIGURE 4: Aromatic region from double-quantum-filtered COSY (500 MHz) of (a) wild-type staphylococcal nuclease, (b) mutant nuclease H46Y, and (c) difference spectrum (wild type − H46Y). Conditions for (a): 3.3 mM protein, 0.3 M NaCl, pH 5.7, 298 K, 512 experiments; each experiment was the sum of 96 transients. The transformed data set was multiplied by a zero-shifted sine-bell function in both dimensions. Interfering noise due to t_1 ridges was removed by using the program MAKEUP (Zolnai et al., 1986). The arrows indicate the position of the extra cross-peak due to Tyr^{46} in the mutant H46Y. Conditions for (b): 3.3 mM protein, 0.3 M NaCl, pH 5.7, 298 K, 512 experiments, each consisting of 80 scans. Data were processed as above. The resonances due to Tyr^{46} are indicated. The sharp peaks in the difference spectrum (c) between 7.9 and 8.7 ppm are from histidines and result from a slight difference between the pH values of the two samples.

existence of at least two minor forms of nuclease. Similar results also are seen with wild type (data not shown).

The peaks located around 8.4 ppm that grow during the course of the titration are due to H_ϵ resonances in the denatured state. In the classical model of the denatured state, the protein is in a random coil, and all of the H_ϵ resonances should have the same chemical shift (McDonald & Phillips, 1969). Note, however, the nondegenerate chemical shifts of these resonances. Three possible explanations for this are worth considering: (1) The denatured state has some residual structure. (2) The effect of neighboring residues in the sequence on the chemical shifts of the H_ϵ resonances is not negligible. (3) The effect of neighboring residues on the pK_a values of histidines is not negligible.

In any case, three distinct resonances are observed. In the mutant H46Y these correspond to histidines: His^8, His^{121}, and His^{124}. In the spectrum of wild-type nuclease, the multiplet is complicated by the signal from H_ϵ^{46}. We were able to take advantage of the simplification afforded by the missing residue His^{46} to assign the three remaining histidines in thermally denatured H46Y. The assignments were obtained by saturating the native H_ϵ resonances in H46Y and observing transfer of saturation to denatured resonances. The results are presented in Figure 7.

Acid Denaturation of H46Y. Figure 8 shows selected points from the acid denaturation of H46Y. The H_ϵ^{46} resonance in wild-type nuclease overlaps the peak due to the denatured state (see Figure 3, pH 4.02). The mutant H46Y is useful because H_ϵ^{46} is removed. Evidence for the existence of a minor conformational form of nuclease at room temperature and low pH is the minor peak assigned to His^8 (peak 8′ in Figure 8). Epstein et al. (1971) found a peak immediately upfield from the H_ϵ resonances, which they were unable to assign. We find this peak, which separates from H_ϵ^{121} below pH 4.8, titrates (see Figures 8 and 5a), and we assign it to a minor resonance due to His^{121}. We refer to this resonance as $H_\epsilon^{121\prime}$. The

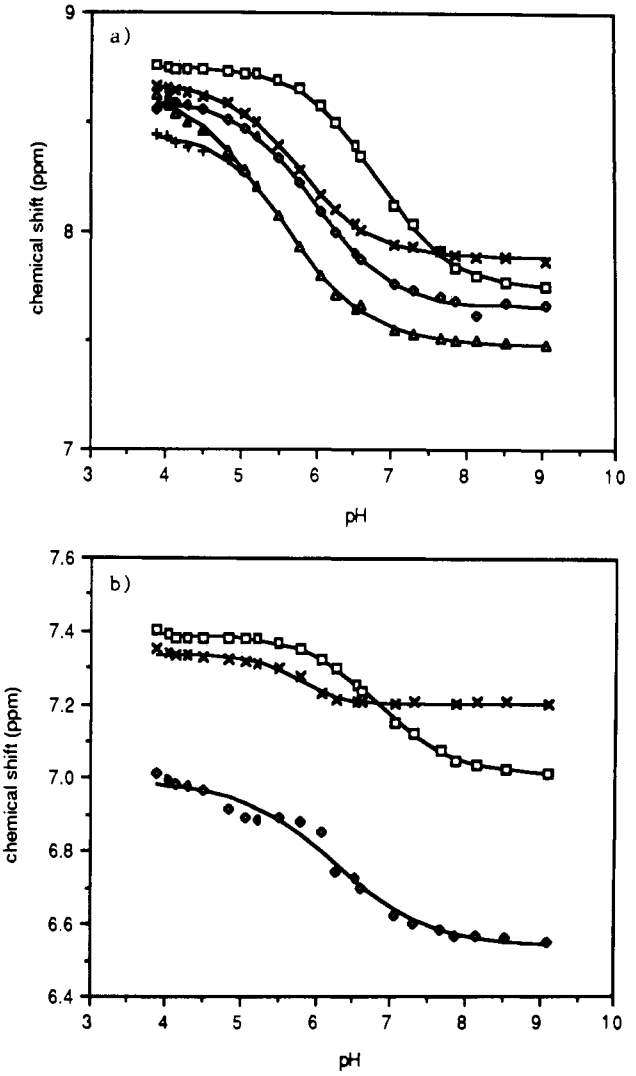

FIGURE 5: Plot of chemical shifts of histidine resonances of staphylococcal nuclease as a function of pH. (a) H_ϵ resonances. Symbols: (□) H_ϵ^8; (×) H_ϵ^{46}; (△) H_ϵ^{121}; (+) $H_\epsilon^{121\prime}$; (◇) H_ϵ^{124}. (b) H_δ resonances. Symbols: (□) H_δ^8; (×) H_δ^{46}; (◇) H_δ^{124}. The points represent the actual data, and the lines represent the least-squares fit to these points. Experimental conditions were as given in the legend to Figure 3.

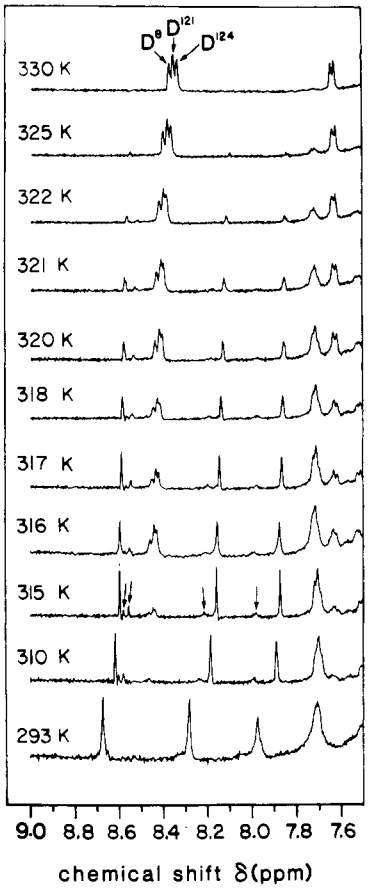

FIGURE 6: Temperature dependence of the 500-MHz ^{1}H NMR spectrum of mutant nuclease H46Y. Conditions: 3.3 mM protein, pH 5.7 at room temperature, 0.3 M NaCl. Each spectrum resulted from the sum of 256 transients. The spectra were multiplied by a $(\pi/25)$-shifted sine-bell function. The arrows in the trace at 315 K indicate resonances due to minor forms. Note the presence of two minor resonances for His^8.

downfield chemical shift as a function of decreasing pH makes it unlikely that it is of non-histidine origin.[2] Although we have not been able to obtain saturation transfer between peaks H_ϵ^{121} and $H_\epsilon^{121\prime}$ at room temperature, presumably because the rate of exchange is too slow, we believe that the resonance is derived from His^{121} because it shows up in wild type, H8R, H46Y, and H124L. What is interesting about $H_\epsilon^{121\prime}$ is that whereas the minor peak $H_\epsilon^{8\prime}$ is very close to the H_ϵ^8 peak (resolution enhancement must be used to resolve the peaks), $H_\epsilon^{121\prime}$ is separated by as much as 0.18 ppm from H_ϵ^{121} at pH 3.88. Figure 5a shows the titration behavior of H_ϵ^{121} relative to that of $H_\epsilon^{121\prime}$. We used the low-pH plateau for $H_\epsilon^{121\prime}$ to calculate a pK_a value for His^{121} in this minor form (see Table I).

Epstein et al. (1971) measured in their acid-induced denaturation of wild-type nuclease the intensity of the H_ϵ resonances as a function of acidity. They found that His^{121} denatures at a slightly higher pH (pK_f = 4.1) than His^8 and His^{124} (pK_f = 3.9). Our results with H46Y suggest that their result may be explained by an increase in the relative population of the minor form characterized by peak $H_\epsilon^{121\prime}$ rather than early denaturation of the region around His^{121}.

CONCLUSIONS

In this paper we have presented new evidence for the assignments of the histidine resonances of staphylococcal nuclease. The results reconfirm previous assignments (Markley, 1969). Since NOE connectivities have been observed between histidine ring protons and the AMX spin systems of two out of three histidines of H124L, these assignments are being used as starting points for NOE-based sequential assignments in nuclease (J. Wang and J. L. Markley, unpublished data).

In principle, one could attempt to assign any amino acid in a protein by creating the appropriate mutant. An obvious drawback to this method is the cost and effort required to make the mutants. In practice, problems will arise in the following situations: (a) The mutation disturbs the structure so much that comparisons to wild type can no longer be made. The nuclease mutation F76V has this effect (Ulrich et al., 1987). (b) The mutation makes the protein susceptible to proteolysis and difficult to purify. The mutations F34Y and H121P have this effect (A. T. Alexandrescu, unpublished results). (c) The resonance to be assigned is in a crowded region of the spectrum. Compare the assignments for the H_ϵ peaks to those for the H_δ and Tyr^{46} peaks. So far, we have used this method only

[2] The sample that was used in Figure 8 contained an impurity peak F whose titration behavior is characteristic of formate. Peaks F and $H_\epsilon^{121\prime}$ are separate and have different titration properties; this rules out formate as the origin of peak $H_\epsilon^{121\prime}$.

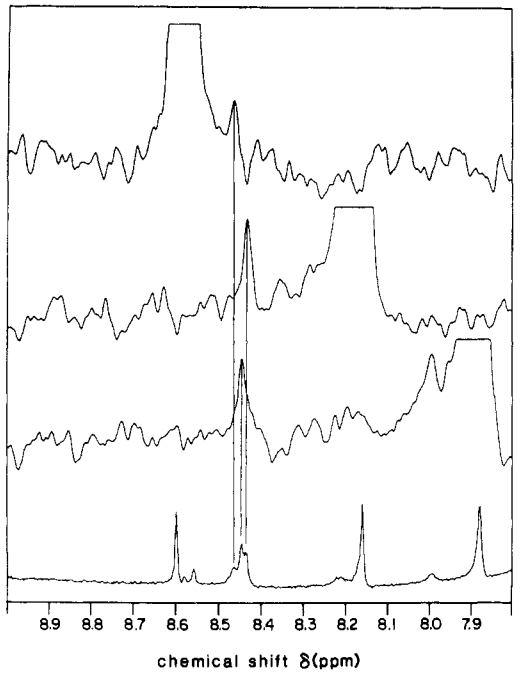

FIGURE 7: Assignment of the three histidines of H46Y in the denatured state. Conditions: 3.3 mM protein, 316 K, 0.3M NaCl, pH 5.7 at room temperature. Spectra were acquired in an interleaved fashion with the decoupler saturating on-resonance or off-resonance from the observed peak in the direction opposite to the saturated peak by an amount equal to the difference between the saturated peak and the observed peak. A PRESAT pulse sequence (Bruker DISNMR software) was used with a D1 delay of 2 s and a decoupler power of 30 μW. The water peak was not presaturated. Bottom to top: blank spectrum in which saturation occurred in a region of the spectrum without any peaks, difference spectrum (blank – saturation on H_ϵ^{121}), difference spectrum (blank – saturation on H_ϵ^{124}), and difference spectrum (blank – saturation on H_ϵ^{8}). All of the difference spectra were multiplied by an exponential function with a line broadening of 5 Hz. Each spectrum was the result of 128 transients.

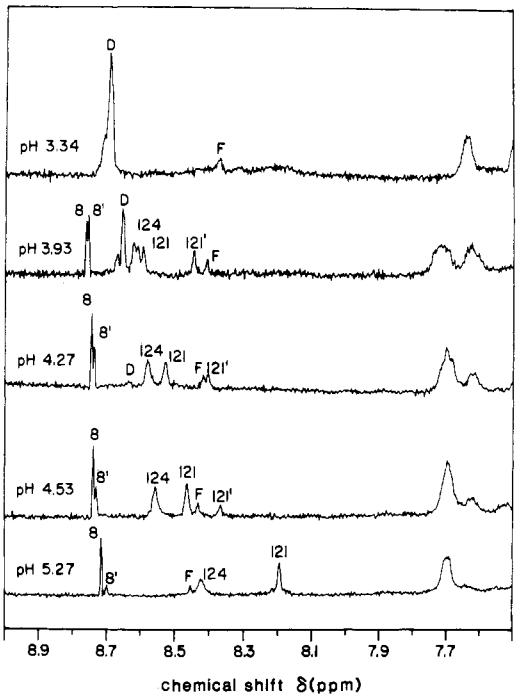

FIGURE 8: Selected points from the acid denaturation of H46Y. Conditions: 1.85 mM protein, 0.3 M NaCl, 298 K. Each spectrum resulted from the sum of 128 transients. Symbols: 8 = H_ϵ^{8}; 8′ = H_ϵ^{8} resonance due to a minor form of nuclease; D = H_ϵ resonance of the denatured form; 124 = H_ϵ^{124}; 121 = H_ϵ^{121}; 121′ = H_ϵ^{121} from the minor form; F = formate (a contaminant). In order to enhance resolution, the spectra were multiplied by a (π/25)-shifted sine-bell function.

to assign aromatic peaks in nuclease. It remains to be seen whether this method can be used for aliphatic residues such as Ile, Leu, Val, and Ala, or if a more sophisticated approach will be necessary to assign those resonances.

It is clear from the data presented in this report that the denaturation of nuclease cannot be described by a simple two-state mechanism. Clearly, the unfolding process is highly cooperative, but the existence of multiple conformations can be demonstrated directly by observing minor resonances. It has been found that the addition of Ca^{2+} and the inhibitor pdTp reduces the intensity of the minor resonances (Fox et al., 1986; Markley et al., 1970). One possibility is that the minor resonances represent a soluble aggregated form of nuclease. This is inconsistent with the concentration dependence of the intensities of these minor peaks (Ulrich et al., 1987). Recently, Evans et al. (1987) have presented evidence linking the conformational heterogeneity observed in native nuclease to cis/trans isomerization of the peptide bond at Pro^{117}.

The heterogeneity of the chemical shifts of the denatured H_ϵ resonances may indicate that the classical random coil model is inadequate for the denatured state of nuclease. We have observed heterogeneity in the denatured resonances from other amino acid types, although we have not assigned them yet. We anticipate that as new techniques become available for improving resolution (such as higher magnetic field strengths) and for making assignments (such as $^{15}N/^{13}C$ labeling), we will obtain a better understanding of the denatured state of proteins. This knowledge will be of paramount importance to understanding protein folding.

ACKNOWLEDGMENTS

We are deeply indebted to Dr. David Shortle for his gifts of strains and plasmids and for useful discussions. We thank Phil Johnson of the University of Wisconsin pilot plant, Jennifer Clough, and Dr. Charles Grissom for assistance. Discussions with John Makris and Vince Schulz, of the W. S. Reznikoff lab, and Carol Kitzinger were of great help in cloning the H8R mutant. We thank A. S. Mildvan for providing manuscripts prior to publication.

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Velocity of the Creatine Kinase Reaction in the Neonatal Rabbit Heart: Role of Mitochondrial Creatine Kinase†

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Received July 6, 1987; Revised Manuscript Received November 2, 1987

ABSTRACT: To examine the role of changes in the distribution of the creatine kinase (CK) isoenzymes [BB, MB, MM, and mitochondrial CK (mito-CK)] on the creatine kinase reaction velocity in the intact heart, we measured the creatine kinase reaction velocity and substrate concentrations in hearts from neonatal rabbits at different stages of development. Between 3 and 18 days postpartum, total creatine kinase activity did not change, but the isoenzyme distribution and total creatine content changed. Hearts containing 0, 4, or 9% mito-CK activity were studied at three levels of cardiac performance: KCl arrest and Langendorff and isovolumic beating. The creatine kinase reaction velocity in the direction of MgATP production was measured with ^{31}P magnetization transfer under steady-state conditions. Substrate concentrations were measured with ^{31}P NMR (ATP and creatine phosphate) and conventional biochemical analysis (creatine) or estimated (ADP) by assuming creatine kinase equilibrium. The rate of ATP synthesis by oxidative phosphorylation was estimated with oxygen consumption measurements. These results define three relationships. First, the creatine kinase reaction velocity increased as mito-CK activity increased, suggesting that isoenzyme localization can alter reaction velocity. Second, the reaction velocity increased as the rate of ATP synthesis increased. Third, as predicted by the rate equation, reaction velocity increased with the 3-fold increase in creatine and creatine phosphate contents that occurred during development.

Creatine kinase (EC 2.7.3.2, creatine phosphotransferase), present in high activity in heart, catalyzes the reaction MgADP + creatine phosphate ↔ MgATP + creatine. In heart, there are four creatine kinase isoenzymes: MM, MB, BB, and mitochondrial (mito-CK).[1] Mito-CK, bound on the inner membrane of the mitochondria, and MM-CK, a significant portion of which is localized at sites of ATP utilization, are the predominant isoenzymes in mature myocardium. MB- and BB-CK, which are cytosolic enzymes, are present primarily in immature myocardium.

Controversy persists regarding the role of the creatine kinase in energy transfer in muscle and the determinants of the creatine kinase reaction velocity in vivo. To date, studies using ^{31}P magnetization transfer to measure the creatine kinase reaction velocity in intact hearts have primarily examined the role of substrates in regulating the reaction velocity. While it is accepted that the creatine kinase reaction velocity is severalfold faster than the rate of ATP synthesis by oxidative phosphorylation, the relationship between changes in the rate of ATP synthesis and the creatine kinase reaction velocity remains controversial. Studies looking at wide ranges of cardiac performance, including arrest (Kupriyanov et al., 1984; Bittl & Ingwall, 1985, 1986), have shown that the reaction

† This work was supported by U.S. Public Health Service Grant HL35675. S.B.P. was supported by a Grant-in-Aid from the American Heart Association, Massachusetts Affiliate, Inc., and National Research Service Award HL07309.
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[1] Abbreviations: CK, creatine kinase; CP, creatine phosphate; mito-CK, mitochondrial creatine kinase; Na_2H_2EDTA, disodium ethylenediaminetetraacetate; T_1, longitudinal nuclear relaxation time; RPP, rate-pressure product (product of heart rate times left ventricular developed pressure).

0006-2960/88/0427-2165$01.50/0