Syntheses and NMR Studies of Specifically Labeled [2-^{15}N]Phosphocreatine, [2-^{15}N]Creatinine, and Related ^{15}N-Labeled Compounds

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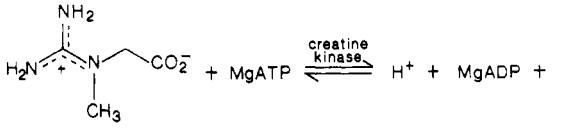
Abstract: [2-^{15}N]Creatine, [2-^{15}N]creatinine, [2/3-^{15}N]creatinine, [2-^{15}N]phosphocreatinine, [2/3-^{15}N]phosphocreatinine, [2-^{15}N]phosphocreatine, and [2/3-^{15}N]phosphocreatine have been synthesized. ^{1}H and ^{15}N NMR analyses of the labeled creatinines have established that creatinine is protonated at low pH exclusively at the unmethylated ring nitrogen (N-3) and indicate that in the predominant resonance form at low pH, conjugation to the carbonyl is present. ^{31}P and ^{15}N NMR analyses of the labeled phosphocreatinines establish that the site of phosphorylation of phosphocreatinine is on the exocyclic nitrogen. The ^{31}P-^{15}N one-bond coupling constant in [2-^{15}N]phosphocreatine is 18 Hz, not 3 Hz as reported for doubly ^{15}N-labeled phosphocreatine by Brindle et al. [Brindle, K. M.; Porteus, R.; Radda, G. K. *Biochem. Biophys. Acta* **1984**, *786*, 18]. Finally, ^{31}P and ^{15}N NMR analyses indicated that no spontaneous ^{15}N/^{14}N-positional isotope exchange occurs in specifically labeled phosphocreatine. The relatively large $J_{^{31}P-^{15}N}$ value and the lack of chemical scrambling are prerequisites for the use of [2-^{15}N]phosphocreatine in positional isotope exchange studies in enzymatic reactions.

Creatine (**1**) and phosphocreatine (**2**) are of great importance in the energy fluxes involved in the muscle contractile process. When muscle ATP levels are high, energy is stored by the reversible creatine kinase-catalyzed transfer of the γ-phosphoryl group of ATP to creatine. The reverse reaction provides a ready source of ATP for muscle activity.



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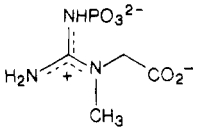

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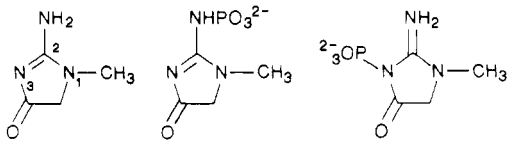
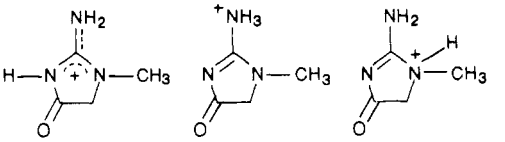
Creatinine (**3**) is an important end product of nitrogen metabolism in vertebrates and appears in the urine of normal adult humans. Creatinine is formed mostly from non-enzymatic cyclization of creatine. However, according to a recent report, non-enzymatic hydrolysis of phosphocreatinine (**4**/**5**) to give creatinine (**3**) may account for a significant fraction of the daily loss of phosphocreatine and creatine from muscle tissue.[1]



3 **4** **5**

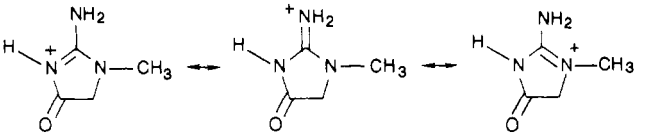
These compounds and numerous analogues have been used extensively in investigations into the mechanism of creatine kinase over the course of the last 75 years.[2-5] The chemical and structural properties of these molecules have been investigated as well. However, there are still unanswered questions about the chemical properties of these molecules and how they interact with creatine kinase. In the investigations reported here we address three questions.

The first question is where creatinine is protonated at low pH. Protonation of creatinine at low pH values could conceivably occur at any one of the three guanidino nitrogens, but the exact site of protonation has never been established.[6,7] It was also possible



6 **7** **8**

that a tautomeric equilibrium existed between two or more of structures **6**, **7**, and **8**. In addition, structure **6** can be represented by three resonance forms:



9 **10** **11**

The second question is whether phosphocreatinine is phosphorylated at N-2 or N-3. Phosphocreatinine (**4**/**5**) is an inhibitor of the reaction catalyzed by creatine kinase with K_i = 5 mM compared to a K_m = 0.5 mM for creatine.[8] However, a related compound, 1-(carboxymethyl)-2-iminoimidazolidine (cyclocreatine) (**12**), is a substrate analogue with a V_{max} 90% of that for creatine (**1**).[2,3] Firm establishment of the sites of phosphorylation in phosphocreatinine and in phosphocyclocreatine is

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(1) Iyengar, M. R.; Coleman, D. W.; Butler, T. M. *J. Biol. Chem.* **1984**, *260*, 7562.
(2) Rowley, G. L.; Greenleaf, A. L.; Kenyon, G. L. *J. Am. Chem. Soc.* **1971**, *93*, 5546.
(3) McLaughlin, A. C.; Cohn, M.; Kenyon, G. L. *J. Biol. Chem.* **1972**, *247*, 4382.
(4) Dietrich, R. F.; Miller, R. B.; Kenyon, G. L.; Leyh, T. S.; Reed, G. H. *Biochemistry* **1980**, *19*, 3180.
(5) Nguyen, A. C. Ph.D. Thesis, University of California, San Francisco, 1984.
(6) The tautomeric preferences of an extensive series of glycocyamidine analogues at pH 12 have been examined by UV spectroscopy.[2,7] For creatinine at pH 12 structure **3** is preferred. In that structure there is conjugation between the guanidinium and carbonyl moieties. At low pH this conjugation would still be expected to predominate. However, at low pH the UV spectra were ambiguous.
(7) Matsumoto, K.; Rapoport, H. *J. Org. Chem.* **1968**, 33, 552.
(8) Gercken, G.; Doring, V. *FEBS Lett.* **1974**, *46*, 87.

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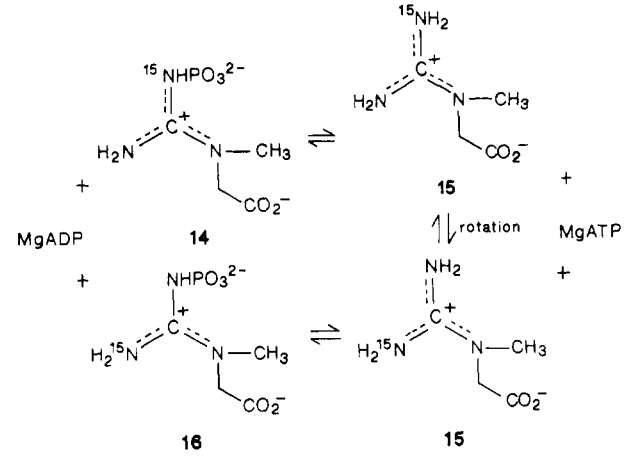
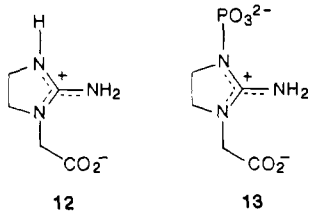
Scheme I



fundamental to understanding why these molecules interact differently from each other in the creatine kinase-catalyzed reaction. The structure of phosphocyclocreatine was established by NMR studies[9] and by X-ray crystallographic determination.[10] Zeile and Meyer[11] have purported to establish the structure of phosphocreatinine by a degradative method. However, this proof is not unequivocal.[12]



The third question is whether or not [2-^{15}N]phosphocreatine (**14**) can be used to probe the stereospecificity and kinetics of the creatine kinase reaction. When phosphocreatine labeled with ^{15}N at N-2 is incubated with ADP and creatine kinase and converted to [2-^{15}N]creatine (**15**) and ATP, the two guanidino-NH_2 groups are capable of equilibration via rotation about the carbon–nitrogen bond. Any phosphocreatine resynthesized will be labeled with ^{15}N at the N-3 position 50% of the time. Thus, [2-^{15}N]-phosphocreatine could be used to study creatine kinase by the technique of positional isotope exchange. Before this technique can be used in studies of creatine kinase, however, two preliminary conditions must be met. The first condition is that the phosphorus resonances of [2-^{15}N]phosphocreatine and [3-^{15}N]phosphocreatine can be resolved. Although doubly labeled [$^{15}N_2$]phosphocreatine has previously been reported, the data on NMR properties such as the ^{31}P–^{15}N one-bond coupling constant were not measured directly.[13] The second prerequisite is that the specifically labeled phosphocreatine does not undergo ^{14}N–^{15}N positional isotope exchange all by itself.[14,15]

Materials and Methods

[^{15}N]Ammonium chloride was purchased from MSD Isotopes in 99% isotopic purity. ATP, ADP, NADH, NADP, sarcosine, glycine, α-ketoglutarate, magnesium acetate, Trizma base, creatine kinase, creatinase, Chelex-100(Na^+), unlabeled creatine, creatinine, phosphocreatinine, and phosphocreatine were the products of Sigma. Iodomethane and $POCl_3$ were purchased from Aldrich. Dowex AG 1-8×(Cl^-) (200–400 mesh) was purchased from Bio-Rad. $POCl_3$ was distilled under vacuum at 37 °C. MeOH was heated at reflux over Mg turnings and distilled just prior to use. Dimethylformamide was shaken with KOH and distilled from CaO. Urease and glutamate dehydrogenase were purchased from Boehringer-Mannheim.

Synthesis of Labeled Compounds. 3-Methyl-4-thiohydantoic acid (17) was synthesized according to the procedure of Rowley et al.[2]

***N*-(Carboxymethyl)-*N*-methyl-*S*-methylisothiuronium hydriodide (18)** was synthesized according to the procedure of Rowley et al.[2]

1-Methyl-2-thiohydantoin (19) was prepared by heating a suspension of 3-methyl-4-thiohydantoic acid (1.77 g, 12 mmol) in 11 mL of concentrated HCl and 3 mL of H_2O for 24 h on a steam bath. White, needle-like crystals (1.3 g, 10 mmol, 83.3% yield) were isolated by filtration, washed with ethanol and mixed hexanes, and used successfully without further purification. Both the ^{1}H NMR (s, δ 4.74; s, δ 3.20) and melting point (227–230 °C) were in good agreement with literature values.[2]

[2-^{15}N]Creatine (20) and [2/3-^{15}N]creatinine (22/23) were both prepared by the reaction of [^{15}N]NH_4Cl (100 mg; 1.83 mmol) and *N*-(carboxymethyl)-*N*-methyl-*S*-methylisothiuronium hydroiodide (**18**) (674 mg; 2.32 mmol) dissolved in 1.75 mL of dry MeOH and triethylamine (880 μL; 4.2 mequiv). The reaction mixture was stoppered, covered with aluminum foil, and stirred for 48 h at room temperature. The product (**20**) precipitated out as a white powder that was filtered and used without further purification.

The [2-^{15}N]creatine (**20**) (196 mg) was heated in a stoppered flask at 100 °C for 24 h in 1.64 mL of concentrated HCl with 0.44 mL of H_2O. Water and HCl were removed under vacuum and the residue was passed through a Dowex anion exchange column in H_2O and dried under vacuum. The product was recovered as a white powder and identified as creatinine (**22/23**) as described above (125.6 mg; 60.6% yield based on [^{15}N]NH_4Cl).

1-Methyl-2-(methylthio)-2-imidazolin-4-one hydroiodide (21) was prepared by a modification of the procedure of Rowley et al.[2] A solution of 1-methyl-2-thiohydantoin (**19**) (650 mg, 5 mmol) in 5 mL of DMF at 0 °C was treated with iodomethane (0.8 mL, 10.6 mmol). The solution was warmed slowly to 35 °C over a 30-min period and stirred for an additional 30 min while the product precipitated as a white powder. Unreacted iodomethane was removed in vacuo in a fume hood, and the product was filtered and washed with ethanol and mixed hexanes. NMR (s, δ 2.81; s, δ 3.33; methylenes probably under either the HOD peak or exchanged as in the literature) and melting point (226–227 °C) were in good agreement with the literature values.[2] Yield: 806 mg; 2.7, mmol, 55.6% of theoretical.

Creatinine (3) (Method I). To 40 mL of a 1 M methanolic NH_3 solution was added freshly prepared 1-methyl-2-(methylthio)-2-imidazolin-4-one·HI (**21**) (14.1 g, 51.8 mmol) and then 6 mL of dry triethylamine. The flask was stoppered, covered with aluminum foil, and stirred at 0 °C until nearly all of the solid had dissolved and the solution had turned dark brown. The reaction mixture was stirred 12 h at room temperature while the product precipitated as a light-colored solid. After an additional 7 h of stirring, the precipitate was filtered and rinsed with $CHCl_3$. The filtrate was shaken with an equal volume of $CHCl_3$, and the resulting precipitate was filtered and rinsed with $CHCl_3$. The Benedict–Behre[16] test gave a positive result with both precipitates. The crude creatinine was passed through a Dowex anion exchange column in H_2O. The eluate was evaporated to dryness, and white crystals of creatinine were obtained (1.1 g, 23.7% yield based on 1 L of gaseous NH_3). The product was identified as creatinine by comparison with commercial samples using both ^{1}H NMR and TLC on silica gel.[17]

(9) Struve, G. E.; Gazzola, C.; Kenyon, G. L. *J. Org. Chem.* **1977**, *42*, 4035.

(10) Phillips, G. N., Jr.; Thomas, J. W., Jr.; Annesley, T. M.; Quiocho, F. A. *J. Am. Chem. Soc.* **1979**, *101*, 7120.

(11) Zeile, K.; Meyer, H. *Hoppe-Seyler's Z. Physiol. Chem.* **1938**, *252*, 101.

(12) Zeile and Meyer employed a similar degradative method to determine that 1,3-dimethyl-2-imidazolidin-4-one is the methylcreatinine isomer produced when creatinine is treated with dimethyl sulfate.[11] Later, it was shown that 1,3-dimethyl-2-imidazolidin-4-one hydrolyzes spontaneously in water to generate the acyclic creatine analogue.[2] Kenyon and Rowley noted that this reaction could provide a pathway for the interconversion of methylcreatinine isomers under the conditions used by Zeile and Meyer. If so, their structure proof would be negated. An independent synthesis of the isomeric methylcreatinine ultimately proved that such an isomerization does not occur in the methylcreatinines. Nevertheless, a similar uncertainty still surrounds the existing structure proof for phosphocreatinine.

(13) Brindle, K. M.; Porteous, R.; Radda, G. K. *Biochem. Biophys. Acta* **1984**, *786*, 18.

(14) Although this process would not seem likely on the basis of examination of molecular models of phosphocreatine, i.e., a four-membered ring transition state would be required for attack of the NH_2 on the phosphoryl group, related intramolecular phosphoryl transfers have been proposed.[15] In the case of phosphoenolpyruvate, an intramolecular phosphoryl transfer requiring a five-membered ring transition state was shown to explain the observation of unusually facile exchanges of the carboxyl and phosphoryl oxygens.

(15) O'Neal, C. C., Jr.; Bild, G. S.; Smith, L. T. *Biochemistry* **1983**, *22*, 611.

(16) Benedict, S. R.; Behre, J. A. *J. Biol. Chem.* **1936**, *114*, 515.

(17) Shoptaugh, N. H.; Buckley, L. J.; Ikawa, M.; Sasner, J. J., Jr. *Toxicon* **1978**, *16*, 509.

Creatinine (Method II). [2-^{15}N]Creatinine (**22**) was prepared by the reaction of 1-methyl-2-(methylthio)-2-imidazolin-4-one hydroiodide (**21**) (4.2 g; 15.4 mmol) and [^{15}N]NH_4Cl (0.5 g; 9.17 mmol) dissolved in 8.5 mL of dry methanol and triethylamine (3.5 mL; 25.1 mmol). The solution was stoppered, covered with aluminum foil, and stirred for 48 h at room temperature. The solution darkened to deep amber within 1 h. After 4 h the crude product which had precipitated as a light-colored powder was filtered (549 mg). Solvent was removed from the filtrate under vacuum, and the residue was stirred with 25 mL of $CHCl_3$ for 15 min. An additional 115 mg of crude creatinine was recovered by filtration. The combined crude products were passed through the Dowex anion exchange column in H_2O. The product was recovered as a white powder (556 mg; 4.9 mmol; 50% yield based on [^{15}N]NH_4Cl) and identified as creatinine by TLC on silica gel (BuOH/HOAc/H_2O 2:1:1)[16] by ^{1}H NMR and by GC–MS of the di-trifluoro-acetate of the 2-hydroxy-2-methylethyl derivative.[18]

[2-^{15}N]Phosphocreatinine (24) was prepared by a method that closely paralleled the procedure of Zeile and Meyer[11] for the phosphorylation of creatine.

To a stirred suspension of [2-^{15}N]creatinine (**22**) (545 mg; 4.3 mmol) in 115 mL of freshly distilled $POCl_3$ was added 79 μL (4 mmol) of H_2O. The suspension was heated at reflux for about 45 min to dissolve the creatinine. Reflux was continued an additional 3 h before the $POCl_3$ was removed under vacuum at 37 °C. Residual $POCl_3$ was removed by repeated evaporation of 10-mL aliquots of anhydrous ether under reduced pressure. Finally, the residue was hydrolyzed and neutralized to a phenolphthalein end point with 4 N NaOH at 0 °C. The sample was purified by anion-exchange chromatography at 4 °C in a modification of the method of Martonosi[19] by using first a 250 mL linear gradient of 0–0.3 M TEA/HCO_3^- (pH 7.8) and then 200 mL of 0.3 M TEA/HCO_3^- as eluent. Fractions were assayed for acid-labile phosphates according to the procedure of Ames[20] and for the guanidinium moiety according to the procedure of Benedict–Behre.[16] Phosphocreatine elutes from the column at 0.25 M TEA/HCO_3^- under these conditions. The pooled fractions were concentrated under vacuum below 30 °C to remove water and buffer and then stored at 0 °C (2.55 mmol; yield 59% based on [2-^{15}N]creatinine). The product was identified by ^{31}P NMR, by TLC on silica gel, and by hydrolysis to form phosphocreatine (**26**).

[2/3-^{15}N]Phosphocreatinine (24/25) was prepared from [2/3-^{15}N]-creatinine (**22/23**) in the same manner as [2-^{15}N]phosphocreatinine (**24**) in a yield of 31.8%.

[2-^{15}N]Phosphocreatine (26) was synthesized by using a modification of the procedure of Iyengar et al.[21] by mild hydrolysis of 320 mL of 8 mM [2-^{15}N]phosphocreatinine (**24**) (2.55 mmol) in 30 mM glycine buffer (pH 9.0). This solution was incubated at 37 °C for 24 h. The sample was purified by anion-exchange chromatography at 4 °C on the Dowex anion exchange column by using first a 250-mL linear gradient of 0–0.3 M TEA/HCO_3^- (pH 7.8) and then 200 mL of 0.3 M TEA/HCO_3^- as eluent. Fractions were assayed for acid-labile phosphates according to the procedure of Ames.[20] Phosphocreatinine is eluted from the column at 0.3 M TEA/HCO_3^- under these conditions. The pooled fractions were concentrated under vacuum below 30 °C to remove water and buffer and then stored at 0 °C (655 μmol, 25.7% yield). The product was identified as [2-^{15}N]phosphocreatine (**26**) by comparison of the ^{31}P chemical shift at pH 9.0 (d, δ 3.1, upfield from a 0.85% H_3PO_4 external reference; J_{NP} = 18 Hz) with that for the unlabeled phosphocreatine, by co-spotting with unlabeled phosphocreatine on silica gel TLC with BuOH/HOAc/H_2O (2/1/1) (R_f 0.34),[16] and by enzymatic reaction with ADP in the reaction catalyzed by creatine kinase. The product was contaminated by a small amount of glycine buffer, but this did not interfere with quantitation, enzymatic reactions, ^{31}P NMR, or ^{15}N NMR spectra.

[2/3-^{15}N]Phosphocreatine (26/27) (Method I) was prepared from [2/3-^{15}N]phosphocreatinine in the same manner as [2-^{15}N]phosphocreatinine in an overall yield of 3.3%.

[2/3-^{15}N]Phosphocreatine (26/27) (Method II) was synthesized by enzyme-catalyzed positional isotope exchange of [2-^{15}N]phosphocreatine (**26**). [2-^{15}N]Phosphocreatine (**26**) (350 μmol) was dissolved in 35 mL of 6.0 mM ADP, 15.2 mM ATP, 20.5 mM $Mg(OAc)_2$, and 50 mM Tris/HOAc. Enzymatic reaction was initiated by the addition of 1225 units of creatine kinase. It was estimated[22,23] that equilibrium would be achieved in 2–3 h after 50% of the initial phosphocreatine was consumed.

(18) Bjorkhem, I.; Blomstrand, R.; Ohman, G. *Clin. Chem.* **1977**, *23*, 2114.
(19) Martonosi, A. *Biophys. Biochem. Res. Commun.* **1960**, *2*, 12.
(20) Ames, B. N. *Methods Enzymol.* **1966**, *8*, 115.
(21) Iyengar, M. R.; Coleman, D. W.; Butler, T. M. *J. Biol. Chem.* **1984**, *260*, 7562.
(22) Lawson, J. W. R.; Veech, R. L. *J. Biol. Chem.* **1979**, *254*, 6528.
(23) Morrison, J. F.; James, E. *Biochem. J.* **1965**, *97*, 37.

Table I. ^{15}N NMR Spectra of ^{15}N-Labeled Compounds

compound	pH	chemical shift (ppm) N-2	chemical shift (ppm) N-3	J_{NP} (Hz)	J_{NH} (Hz)
[2-^{15}N]phosphocreatinine	9.0	84.7		21.4	
[2/3-^{15}N]phosphocreatinine	9.0	84.8	184.6	20.9	
[2-^{15}N]phosphocreatine	9.0	81.2		18.3	
[2/3-^{15}N]phosphocreatine	9.0	81.1	57.3	N.D.[a]	
[2-^{15}N]creatinine	11.0	53.4			
[2-^{15}N]creatinine	2.0	56.3			93.0
[2/3-^{15}N]creatinine	11.0	53.6	180.3		
[2/3-^{15}N]creatinine	2.0	55.9	122.9		93.0
[2-^{15}N]creatine	7.0	56.5		N.D.[a]	

[a] N.D. = not detectable.

After 8 h, the reaction mixture was diluted to 112 mL with H_2O and poured over a 117-mL slurry of 25% acid-washed charcoal in 91 mL of H_2O and filtered through Celite. The sample was purified by anion-exchange chromatography at 4 °C by using a 250-mL linear gradient of 0.15–0.3 M TEA/HCO_3^- (pH 7.8) and then 200 mL of 0.3 M TEA/HCO_3^- as eluent. Fractions were assayed for acid-labile phosphates according to the procedure of Ames.[20] Phosphocreatine elutes from the column at 0.25 M TEA/HCO_3^- under these conditions. The pooled fractions were concentrated under vacuum below 30 °C to remove water and buffer and stored at −20 °C. Yield: 75 μmol.

NMR Sample Preparation. [2-^{15}N]Creatine (**20**), [2-^{15}N]creatinine (**22**), and [2/3-^{15}N]creatinine (**22/23**) were dissolved in 0.4 mL of 20% D_2O to give solute concentrations of 0.08, 0.55, and 0.24 M, respectively. The pH of each solution was adjusted with NaOH and/or HCl.

[2-^{15}N]Phosphocreatinine (**24**), [2/3-^{15}N]phosphocreatinine (**24/25**), [2-^{15}N]phosphocreatine (**26**), and [2/3-^{15}N]phosphocreatine (**26/27**) were dissolved in 0.4 mL of 20% D_2O to concentrations of 80 mM to 1 M, and the pH was adjusted with NaOH.

When necessary, the ^{15}N-labeled samples were percolated through columns of Chelex-100 (sodium) into acid-washed vials and lyophilized to prepare samples of 50–200 mM in 0.4 mL of 20% D_2O. The 5-mm NMR tubes were made metal-free by soaking overnight in 1/1 concentrated HNO_3/concentrated H_2SO_4 and rinsing thoroughly with distilled, deionized water.

NMR Measurements. ^{15}N NMR spectra were obtained at 24.426 MHz on the UCSF wide-bore 240-MHz spectrometer. A spectral width of 6300 Hz and either 8192 or 16384 data points were used to acquire the free induction decay; a 4 s time delay between pulses was employed in most cases. An exponential line-broadening factor of 2–10 Hz was applied to the total free induction decay prior to Fourier transformation. Up to 10 000 data acquisitions were collected. Exponential line broadening of 10–20 Hz was applied in obtaining the spectra for [2-^{15}N]-creatine (**20**) and for [2/3-^{15}N]phosphocreatine (**26/27**) as the low concentrations made it impossible to obtain good signal to noise in a reasonable time period. No proton decoupling was used. Chemical shifts were measured relative to [^{15}N]NH_4NO_3, pH 2.0, used as an external standard.

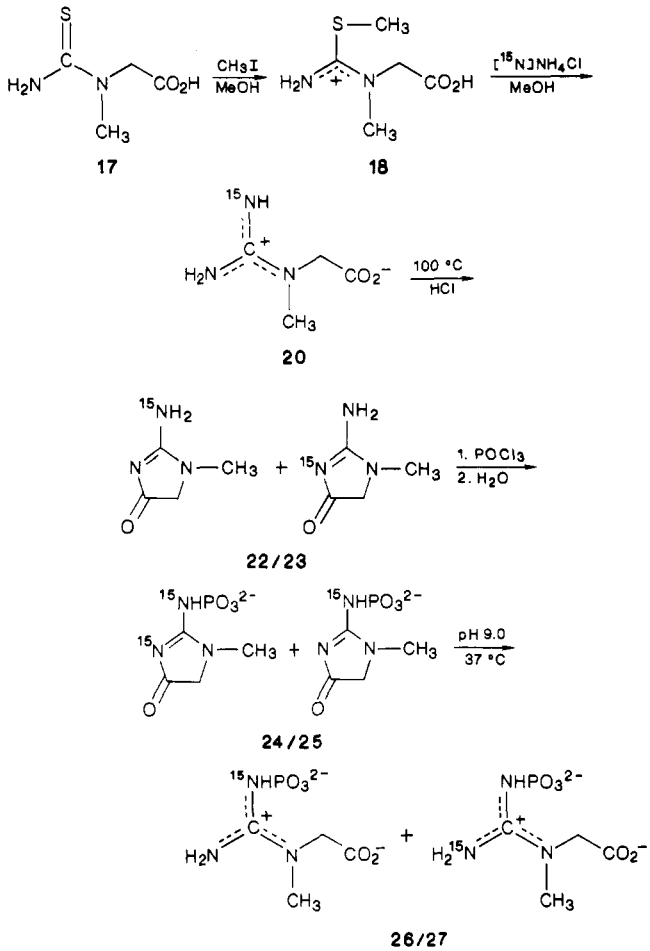
^{31}P NMR spectra were taken at 97.5 MHz on the UCSF wide-bore 240-MHz spectrometer. A spectral width of 2000 Hz and 8196 data points were used to acquire the free induction decay; a 44° tip angle and no time delay between acquisitions were employed in most cases. An exponential line-broadening factor of 0.5–2.0 Hz was applied to the free induction decay prior to Fourier transformation. When necessary, a double exponential apodization factor of up to 10 was applied to the free induction decay prior to Fourier transformation to improve resolution for peak integration. Chemical shifts were measured relative to an 0.85% solution of H_3PO_4 (in D_2O) as external standard. Typically, 500–2000 data acquisitions were collected.

Routine ^{1}H NMR spectra in 99% D_2O were taken at 80 MHz on a Varian FT-80 spectrometer. High-resolution ^{1}H NMR spectra in 20% D_2O were measured at 500.04 MHz on a General Electric 500-MHz spectrometer. A spectral width of 6000 Hz and 8194 data points were used to acquire the free induction decay. A 1:3:3:1 solvent suppression sequence[24] with a tip angle of 25° and a τ value of 370 μs was used. There was no delay between acquisitions. Typically, four data acquisitions were collected.

Preparation of GC–MS Samples of Creatinines. The derivatization of creatinine for GC–MS analysis was as described by Bjorkhem et al.[18] One milligram of creatinine in 0.5 mL of MeOH was treated with 0.5 mL of 1,2-epoxypropane and incubated at 70 °C for 30 min in a Teflon-sealed screw-capped vial. The solvent was evaporated under $N_{2(g)}$,

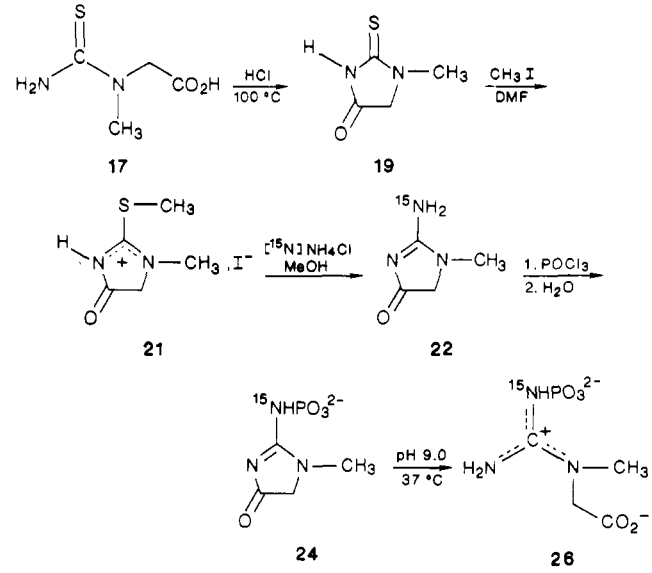
(24) Hore, P. J. *J. Magn. Reson.* **1983**, *55*, 283.

Scheme II



17 18 20 22/23 24/25 26/27

Scheme III



17 19 21 22 24 26

and then the residue was dissolved in 200 μL of EtOAc and treated with 100 μL of trifluoroacetic anhydride for 30 min at 37 °C. The solvent and excess reagent were evaporated under $N_{2(g)}$, and the residue was taken up in 1 mL of mixed hexanes for GC–MS analysis of the derivatized samples on the UCSF MS-25 mass spectrometer.

Results and Discussion

General Characterization of ^{15}N-Labeled Creatine, Creatinine, Phosphocreatine, and Phosphocreatinine by ^{15}N, ^{31}P, and ^{1}H NMR. The nitrogen resonances of unprotonated amides are usually in the 200–230-ppm range.[25] However, the N-3 resonances for phosphocreatinine and creatinine at high pH are 184.6 and 180.3 ppm, respectively (Table I). This is apparently due to the fact that the nitrogens of phosphocreatinine and creatinine are not simple amides but possess guanidino character as well. The amide characters of the N-3 nitrogens of creatinine and phosphocreatinine are lost upon ring opening. Thus, the nitrogen resonance for N-2 of creatine appears over 120 ppm upfield as compared to the resonance for N-3 in creatinine the ring-opened product. The same type of shift in N-3 nitrogen resonances accompanies the ring-opening of phosphocreatinine to form phosphocreatine. Ring-opening does not alter the chemistry at the N-2 position so dramatically in either creatinine or phosphocreatinine. Thus it is not surprising that the upfield change in ^{15}N chemical shift at N-2 accompanying each transformation is less than 5 ppm. The chemical shifts of N-2 in creatine and N-3 in phosphocreatine are close to those observed for other zwitterionic guanidines.[26,27]

Phosphoguanidines have not previously been characterized by ^{15}N NMR. A comparison of the N-2 chemical shifts of creatine and phosphocreatine and of creatine and phosphocreatinine (Table I) shows that phosphorylation of a guanidino nitrogen produces a downfield shift of about 24 ppm in the ^{15}N resonance. In contrast, the resonances of the guanidino nitrogens not directly phosphorylated shift less than 1 ppm.

Isotope-induced chemical shift changes, such as the ^{18}O-induced shift of about 0.02 ppm per ^{18}O on ^{31}P resonances, are widely known.[28,29] The ^{31}P NMR spectra of [2/3-^{15}N]phosphocreatine and [2/3-^{15}N]phosphocreatinine (Figure 3) illustrate that ^{15}N produces an upfield chemical shift change of 0.01 ppm on the ^{31}P resonances of phosphoguanidines as well. In each case the phosphorus resonance appears as a triplet formed by the superposition of the singlet due to ^{31}P bound to ^{14}N upon the doublet due to ^{31}P bound to ^{15}N. The apparent triplets are slightly asymmetric because of the ^{15}N induced chemical shift change. As expected, no such chemical shift change was detected for ^{1}H resonances of protons bonded directly to ^{15}N.[30]

In the ^{1}H NMR spectrum of [2-^{15}N]creatinine (Figure 2A), the signal for the guanidino-NH_2 protons at 8.5 ppm appears as a doublet as a result of the one-bond ^{15}N–^{1}H coupling constant

Table II. ^{31}P NMR Spectra of ^{15}N-Labeled Molecules

compound	chemical shift (ppm) ^{14}N–P	chemical shift (ppm) ^{15}N–P	J_{NP} (Hz)	isotope-induced chemical shift changes
[2-^{15}N]phosphocreatinine	−4.03	−4.04 (minor)	20.2	0.01
[2/3-^{15}N]phosphocreatinine	−4.08	−4.09	20.2	0.01
[2-^{15}N]phosphocreatine	−3.26	−3.27 (minor)	18.2	0.01
[2/3-^{15}N]phosphocreatine	−3.27	−3.28	18.3	0.01

Table III. ^{1}H NMR Spectra of ^{15}N-Labeled Creatine and Creatinines

compound	pH	chemical shift (ppm)	J_{NH} (Hz)
[2-^{15}N]creatinine	2	8.5	93
[2/3-^{15}N]creatinine	2	8.5	93
[2-^{15}N]creatine	7	6.8	92
impurity in [2/3-^{15}N]creatinine	2	6.7	92

(25) Witowski, M.; Webb, G. A., Eds. *Nitrogen NMR*; Plenum Press: New York, 1973; p 227.

(26) The nitrogen chemical shifts in argininosuccinate are 69 and 51 ppm for the substituted (N-3) and unsubstituted (N-2) nitrogen resonances, respectively.[27]

(27) Raushel, F. M.; Garrard, L. J. *Biochemistry* **1984**, *23*, 1791.

(28) Cohn, M. *Annu. Rev. Biophys. Bioeng.*, "^{18}O and ^{17}O Effects on ^{31}P-NMR as Probes of Enzymatic Reactions of Phosphate Compounds", 1982.

(29) The magnitude of the chemical shift change depends upon the size of the fractional change in mass upon isotopic substitution.[28] Therefore, the chemical shift change induced by ^{15}N on phosphorus resonances should be less than 0.02 ppm per ^{15}N–^{31}P.

(30) The magnitude of the chemical shift change is also a function of the range of shifts of the resonant nucleus.[28] For example, the chemical shift ranges for ^{31}P and ^{15}N are about 320 and 1000 ppm, respectively.[25]

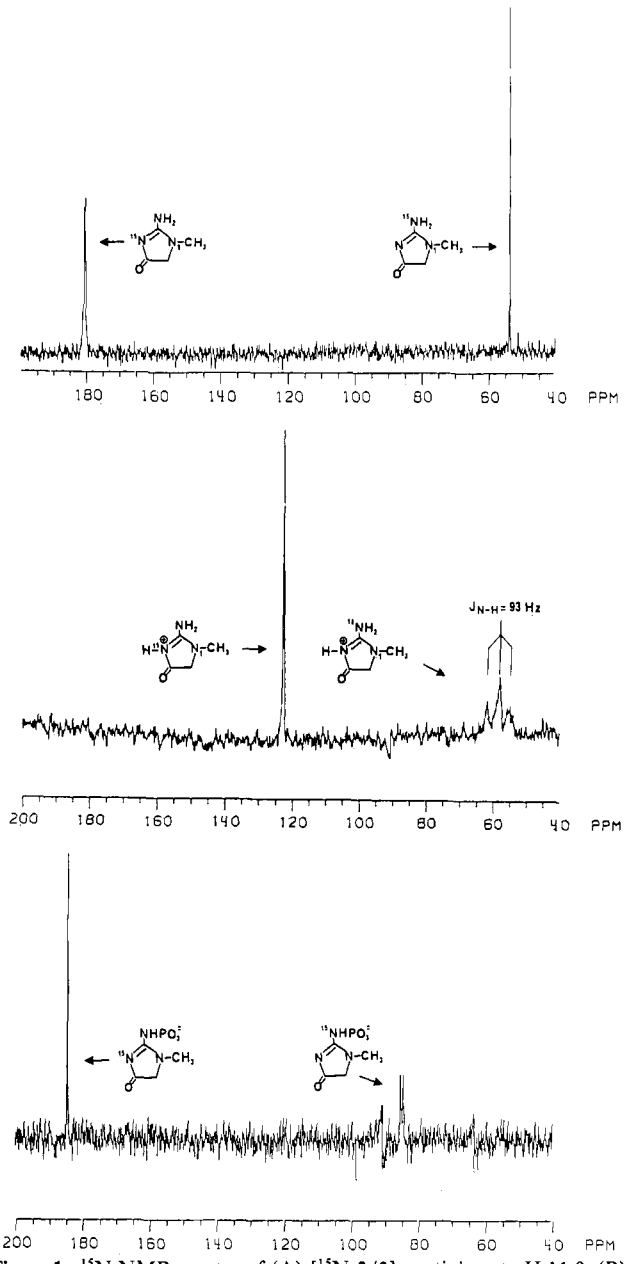


Figure 1. ^{15}N NMR spectra of (A) [^{15}N-2/3]creatinine at pH 11.0, (B) [^{15}N-2/3]creatinine at pH 2.0, and (C) [^{15}N-2/3]phosphocreatinine at pH 9.0.

of 93 Hz. The resonance for protons bound to N-2 in [2/3-^{15}N]creatinine (Figure 2B) is an apparent triplet formed by the superposition of the singlet due to protons bonded by ^{14}N at N-2 upon the doublet due to protons bonded to ^{15}N at N-2.[31] The ^{1}H resonance of the protons bound to ^{14}N (center peak) is obviously much broader than that for the protons bound to ^{15}N (outer peaks). This is due to the large line-broadening effect of the electric quadrupole moment of the ^{14}N nucleus.

The sample of [2/3-^{15}N]creatinine contained a small amount of material giving an apparent triplet at 6.8 ppm, similar to the pattern at 8.5 ppm. This impurity was identified as [2-^{15}N]creatine by comparison of the ^{1}H NMR spectrum. The possibility that these peaks were due to any protons bound to N-3 was ruled out because the intensity of this apparent triplet was less than one-half the intensity of the resonance at 8.5 ppm for protons bound to

(31) Pronounced line broadening of the resonance of ^{14}N is produced by the presence of an electric quadrupole moment in that nucleus. The same quadrupole moment can also produce line broadening in the spectra of the neighboring nuclei.[25] Because of this effect of the electric quadrupole, the resonance of ^{1}H bound to ^{14}N would be expected to be much broader than the same proton bound to ^{15}N.

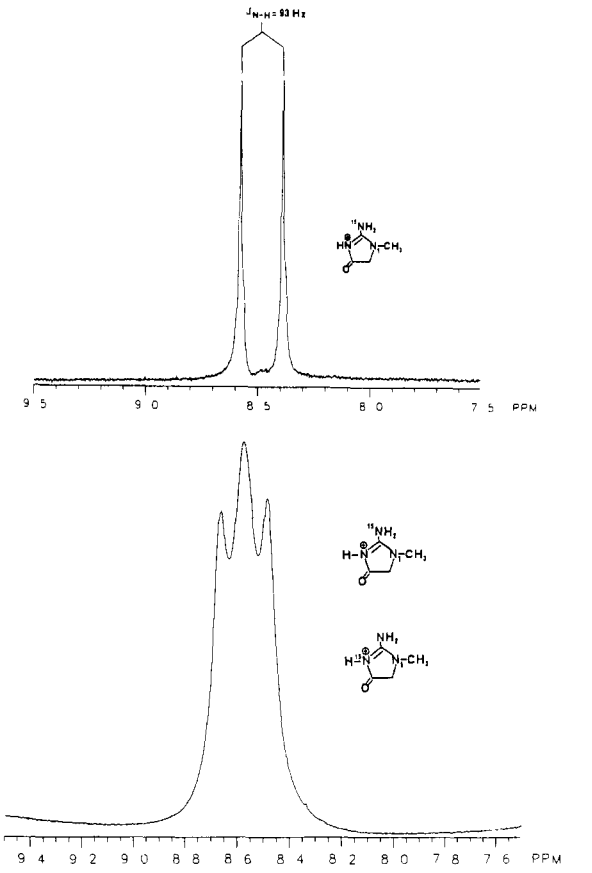


Figure 2. ^{1}H NMR spectra of (A) [^{15}N-2]creatinine at pH 2.0 and (B) [^{15}N-2/3]creatinine at pH 2.0.

N-2 of [2/3-^{15}N]creatinine. Even when the 1:3:3:1 pulse sequence was altered so that the relative signal at 6.8 ppm was maximized, the intensity remained much smaller.

Determination of the Structure of Protonated Creatinine. The protonation of creatinine could conceivably occur at any one of the three guanidino nitrogens present. Two of these tautomers, **6** and **7**, allow conjugation with the carbonyl moiety, but for only one, **6**, is it possible to draw resonance forms for the guanidinium ion. The ^{15}N chemical shift data can be used to firmly establish the site of protonation in this case.[32-34]

The creatinine N-2 resonance is shifted slightly downfield by approximately 3 ppm upon protonation, but the resonance of N-3 shifts upfield nearly 60 ppm (Table I). The large upfield chemical shift change at N-3 is consistent only with protonation at N-3 (**6**), since protonation at either N-1 or N-2 (**7** and **8**) would not be likely to produce much change in the chemical shift of N-3. Thus our results show that creatinine is protonated primarily at N-3 (**6**).

The question remained whether N-3 of creatinine is the exclusive protonation site. The shift change observed for N-3 is somewhat less than is observed for other nitrogen protonations, e.g., pyridine. In addition, a small downfield shift in the N-2 resonance was observed as well. These facts could be interpreted to mean either that N-3 is not the exclusive site of protonation or that the formal positive charge on the N-3 nitrogen (**9**) is distributed partly by resonance to N-1 and N-2.[35] That is, the structure of protonated creatinine may contain contributions from either tautomer (**7**) or resonance from (**10**). Further analysis of

(32) Markowski, V.; Sullivan, G. R.; Roberts, J. D. *J. Am. Chem. Soc.* **1977**, *99*, 714.

(33) Cain, A. H.; Sullivan, G. R.; Roberts, J. D. *J. Am. Chem. Soc.* **1977**, *99*, 6423.

(34) Gonnella, N. C.; Nakanishi, H.; Holtwick, J. B.; Horowitz, D. S.; Kanamori, K.; Leonard, N. J.; Roberts, J. D. *J. Am. Chem. Soc.* **1983**, *105*, 2050.

(35) Parallel arguments have been used in the study of the protonation site of adenosine.[33]

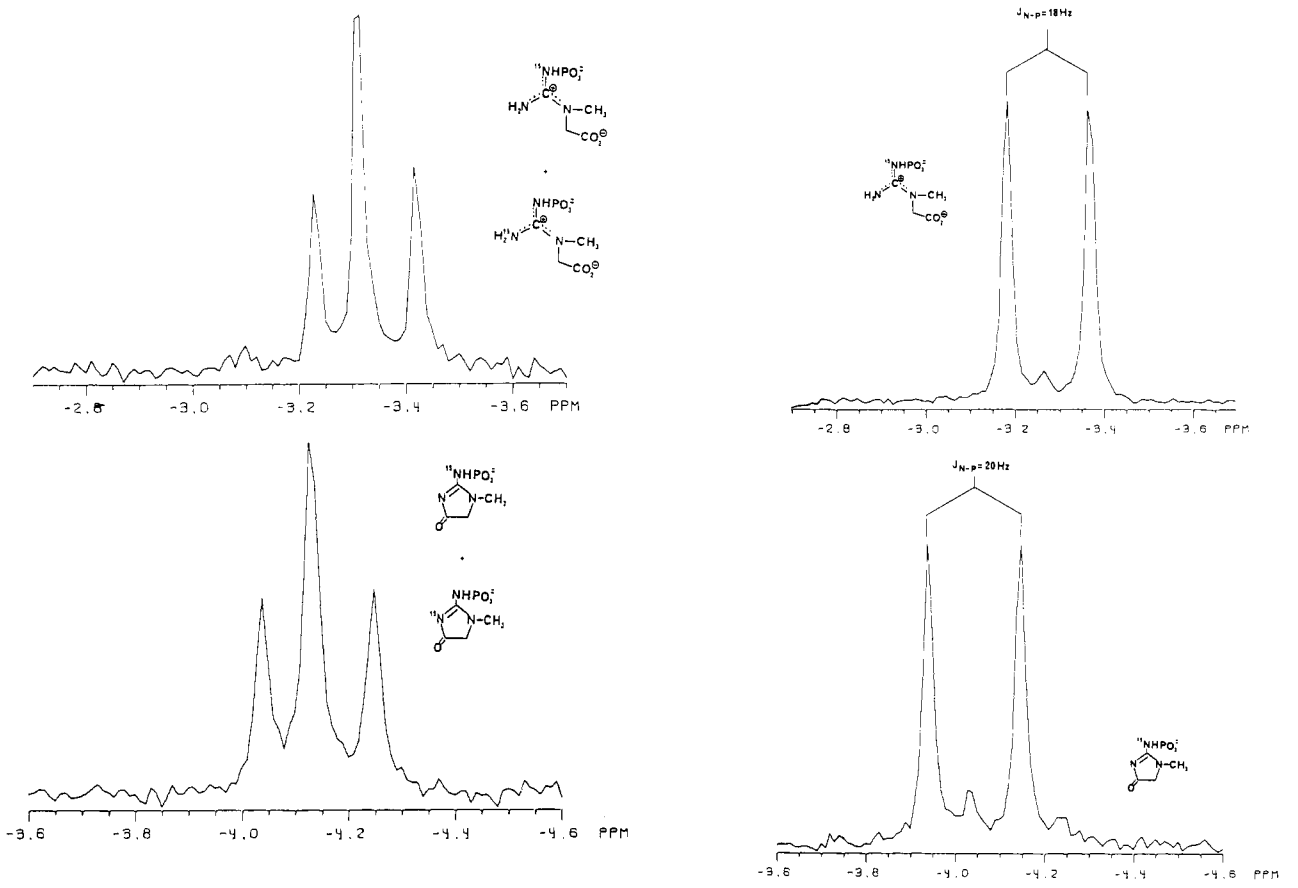


Figure 3. ^{31}P NMR spectra of (A) [^{15}N-2/3]phosphocreatine, (B) [^{15}N-2]phosphocreatine, (C) [^{15}N-2/3]phosphocreatinine, and (D) [^{15}N-2]-phosphocreatinine.

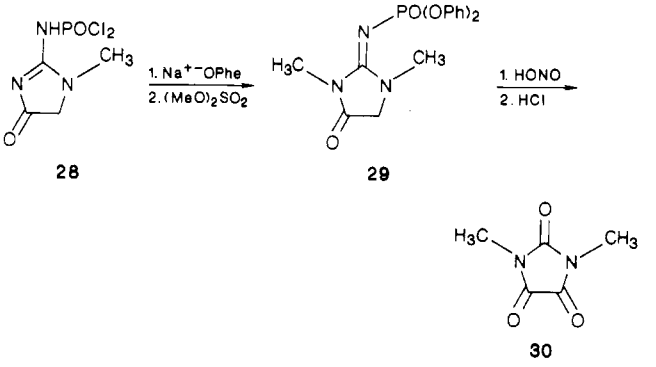
the ^{15}N and ^{1}H NMR spectra of the ^{15}N-labeled creatinines enabled us to make the distinction between these two possibilities by ruling out a tautomeric equilibrium between forms **6** and **7**.

At higher pH, the resonances of N-2 and N-3 (at 56 and 123 ppm, respectively) are singlets (Figure 1A). At low pH, the N-2 resonance is split into a triplet by protons directly attached as a result of the one-bond ^{15}N–^{1}H coupling constant of 93 Hz, but the N-3 resonance remains a singlet (Figure 1B). This heteronuclear coupling is also evident in the ^{1}H NMR spectra for both labeled creatinines (Figure 2, A and B). Thus, there are exactly two protons bound to N-2 and they are in slow exchange with solvent. The ^{1}H resonance of the N-3 proton was presumably too broad to be detected, which indicates that the protons bound to N-3 are in rapid exchange with solvent. These findings are consistent with our earlier conclusion that protonation occurs at N-3. They also establish that tautomer **7** makes no contribution to the structure of protonated creatinine since that tautomer would have three, rather than two, protons bound to N-2. Moreover, these three protons would undoubtedly be in rapid exchange with solvent and thus would be likely to be observed in the ^{1}H spectrum. Therefore, a contribution by resonance form **10** is the most likely explanation for the small downfield shift in the N-2 observed when creatinine is protonated.

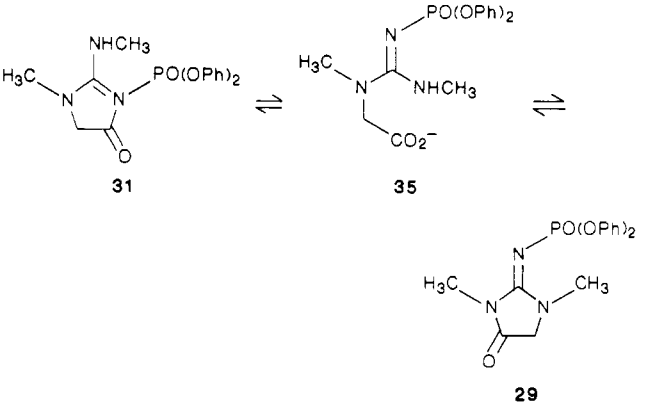
A major contribution to the structure of protonated creatinine by resonance form **10** is certainly ruled out. First, the magnitude of the chemical shift change in the ^{15}N resonance at N-3 is about 67% of the 97-ppm shift observed for the protonation of pyridine.[32] Second, the two protons attached to N-2 are isochronous (not resolved at 500 MHz). This is probably the result of rapid rotation about the C–N2 bond. Such a rotation would be impossible if the double bond character of this bond were high. Thus, resonance form **9** predominates.

Determination of the Structure of Phosphocreatinine. The degradative structure proof for phosphocreatinine reported by Zeile and Meyer is outlined in Scheme IV. If phosphocreatinine were phosphorylated at N-3, the isomeric methyldiphenylphosphocreatinine (**31**) shown in Scheme V would be an additional intermediate in the degradative proof. This intermediate might

Scheme IV



Scheme V



undergo the isomerization outlined in Scheme V. If so, the isomer with an exocyclic phosphoryl group (**29**) would be produced and dimethylparabanic acid (**30**) would still be the end product of the degradative pathway. Therefore, the existing structure proof for phosphocreatinine is open to question. We report the unequivocal determination of the site of phosphorylation in this molecule, based on comparison of the ^{15}N and ^{31}P NMR spectra of phosphocreatinine specifically enriched in ^{15}N at N-2 with those of phosphocreatinine enriched in ^{15}N at N-3.

Upon phosphorylation of [2-^{15}N]creatinine to give [2-^{15}N]-phosphocreatinine, the N-2 resonance is shifted downfield by approximately 30 ppm and is a doublet due to the one-bond ^{15}N–^{31}P coupling constant of 21 Hz (Table I). In contrast, the resonance of N-3 is shifted downfield by only approximately 5 ppm and remains a singlet. Since the large chemical shift change in the ^{15}N resonance and the ^{31}P–^{15}N coupling are observed exclusively for N-2 and not for N-3, the correct structure for phosphocreatinine is that where phosphorus is bonded directly to N-2. The ^{31}P NMR spectra of [2-^{15}N]phosphocreatinine and [2/3-^{15}N]phosphocreatinine (Figure 3, C and D) provide corroborating evidence. The 20-Hz one-bond heteronuclear coupling between ^{15}N and ^{31}P is observed only when ^{15}N is at N-2. This confirms unequivocally the conclusions of Zeile and Meyer.[11]

Implications for Structure–Activity Relationships in the Creatine Kinase Reaction. The lack of reactivity for phosphocreatinine and creatinine as substrates for creatine kinase has been attributed to the lack of a carboxyl moiety in these molecules rather than on any other differential properties with the normal substrate.[8] However, the results of the structural studies on creatinine and phosphocreatinine reported here suggest that the lack of a carboxyl moiety may not be the only explanation. The lack of reactivity in creatinine could instead be attributed to unfavorable positioning of the exocyclic nitrogen (N-3) and to lower nucleophilicity of the nitrogens conjugated to the ring carbonyl (N-2 and N-3). The site of phosphorylation of phosphocreatinine (**4**) is opposite to the analogous site of phosphorylation in phosphocyclocreatine (**13**). In addition, because N-3 of creatinine is conjugated to the carbonyl at high pH and is protonated at low pH it is unlikely to act as nucleophile to attack the 1-phosphoryl of ATP. Thus, it is not possible to infer that the carboxyl group is required for substrate activity from enzyme studies of creatinine and phosphocreatinine alone. In addition, all analogues of creatine examined to date possess either a negatively charged group or a carbonyl group adjacent to one of the guanidino nitrogens.[2-5,8,36] The apparent dependence of activity on the stereochemical position of the carboxyl group[2-4] might be produced by steric effects alone. Further, substitution of a carboxyl group for the methyl of creatine (as in 1-(carboxymethyl)-2-iminoimidazolidin-4-one) does not restore substrate activity lost on cyclization. Thus, the conclusion that the carboxyl group is necessary for substrate activity remains to be established unequivocally.

Feasibility of Using [2-^{15}N]Phosphocreatine To Study the Mechanism of the Creatine Kinase Reaction. The first prerequisite for the use of specifically labeled [2-^{15}N]phosphocreatine as a probe of the creatine kinase-catalyzed reaction is that the relative amounts of phosphocreatine labeled at N-2 and at N-3 can be quantitated. If the ^{31}P resonances of [2-^{15}N]phosphocreatine (**8**) and [3-^{15}N]phosphocreatine (**10**) are resolved, then ^{31}P NMR spectroscopy can be used to quantitate them. Recently, Brindle et al. measured J_{NP} for [2,3-$^{15}N_2$]phosphocreatine by an indirect method and reported it to be 3 Hz.[13] In addition, an isotope-induced chemical shift change in the ^{31}P NMR resonance in [2-^{15}N]phosphocreatine of about 0.01 ppm (1 Hz at 97 MHz) was expected. This would make the line widths necessary for resolution of the apparent triplet in the ^{31}P NMR spectrum of partially scrambled material well under 0.25 Hz. With presently available instrumentation such resolution would be virtually impossible to achieve, especially since the center peak would be broadened by the ^{14}N quadrupolar nucleus bound to the phosphorus of [3-^{15}N]phosphocreatine. Fortunately, however, spectra A and B in Figure 3 show that this coupling constant is 18 Hz. Thus, this analogue is useful for the study of the creatine kinase-catalyzed reaction.

A thorough examination of the indirect method used by Brindle et al.[13] for measuring J_{NP} has revealed that the correct coupling constant of 18 Hz could not have been detected under their experimental conditions. There is no evidence from the spectrum of the [2/3-^{15}N]phosphocreatine to suggest long-range ^{31}P–^{15}N coupling between the phosphorus and N-3 on the order of 3 Hz. Further, the discrepancy between our observations and the report of Brindle et al.[13] cannot be explained by pH effects since the coupling constant was found to be pH independent over the range 5.5–11.0.

The second prerequisite for the use of specifically labeled [2-^{15}N]phosphocreatine (**14**) as a probe of the creatine kinase-catalyzed reaction is that $^{14}N/^{15}N$ positional isotope exchange must not occur by itself.[37] Therefore, it was necessary to determine whether intramolecular transfer of the highly reactive phosphoryl group of phosphocreatine would occur in aqueous solution. Only specific labeling of the phosphoramide nitrogen (N-2) would allow us to find whether such a reaction occurs.

At pH 9.0, the phosphorus resonance in [2-^{15}N]phosphocreatine is a doublet at 4.0 ppm as the result of the one-bond ^{15}N–^{31}P coupling constant of 18 Hz (Figure 3B). A small peak, which integrates for 6 ± 3% of the total peak area, appears slightly off center of the doublet. Thus, some scrambled material is present in the product. However, a small peak in the spectrum of [2-^{15}N]phosphocreatinine (Figure 3D), which integrates for 6 ± 3% of the total peak area, also appears slightly off center of the doublet. This means that the same amount of scrambled material is present in the [2-^{15}N]phosphocreatinine from which the [2-^{15}N]phosphocreatine was prepared. Therefore, the requirement that $^{15}N/^{14}N$ positional isotope exchange does not occur in [2-^{15}N]phosphocreatine (**26**) by itself is met.

The ultimate source of the scrambling is most likely ring-opening and ring-closure of [2-^{15}N]creatinine under the conditions of its synthesis and isolation. Even at very low pH there is some creatine in equilibrium with creatinine. Such an equilibration would eventually lead to complete scrambling of the label. In fact, the spectrum exhibited a small broad peak in the center of the doublet due to the presence of ^{14}N at N-2 (Figure 2A),[38] and there was no evidence of a detectable amount of [2-^{15}N]creatine in the sample. The isotopic purity of the [2-^{15}N]creatinine was shown to be 99.0 ● 0.5% by GC–MS analysis of a creatinine derivative. Therefore, the material containing ^{14}N at N-2 was [3-^{15}N]-creatinine formed by the scrambling of [2-^{15}N]creatinine.

Complete scrambling of [2-^{15}N]phosphocreatine was catalyzed by creatine kinase in the presence of MgADP. When phosphocreatine labeled with ^{15}N at N-2 is converted to creatine and ATP, the two guanidino-NH_2 groups may equilibrate via rotation about the carbon–nitrogen bond. The phosphocreatine resynthesized is labeled with ^{15}N at the N-3 position 50% of the time. This enzymatic scrambling can be used as an alternate synthetic route to [2/3-^{15}N]phosphocreatine. The question of whether the enzyme-catalyzed scrambling results exclusively from rotation of free creatine rather than enzyme-bound creatine is the subject of current investigations.[37]

Conclusions

A synthetic method for the preparation of ^{15}N-labeled phosphocreatine, creatine, creatinine, and phosphocreatinine specifically labeled at N-2 has been developed. A method was also developed for the preparation of analogues in which the ^{15}N label is completely scrambled between the N-2 and N-3 positions. The synthesis of specifically labeled creatinine is accompanied by a small amount of scrambling. We believe this is the result of a small amount of opening and reclosure of the creatinine ring during

(36) Deitrich, R. F.; Kenyon, G. L. *Bioorg. Chem.* **1984**, *12*, 221.

(37) Reddick, R. E.; Kenyon, G. L., manuscript in preparation.

(38) Accurate integration is difficult because the resonance of these protons is broadened by the quadrupolar ^{14}N nucleus and because the use of the 1:3:3:1 pulse sequence leads to distortion of peak phase and intensity in a chemical shift dependent way.

its synthesis and workup. No further scrambling was observed in the syntheses of phosphocreatinine and phosphocreatine from the [^{15}N]creatinine. A notable exception is the alternate route to the synthesis of [2/3-^{15}N]phosphocreatine. This route is the enzyme-catalyzed ^{15}N/^{14}N positional isotope exchange of [2-^{15}N]phosphocreatine by creatine kinase in the presence of MgADP. The mechanism for this scrambling of the label in phosphocreatine involves ^{15}N-labeled creatine as an intermediate.

As expected, the ^{31}P NMR data reflect an ^{15}N-induced chemical shift change of 0.01 ppm. No such change was observed in the ^{1}H resonances of protons bound to N-2 of creatinine. The line-broadening effect of the ^{15}N quadrupolar nucleus is evident in comparing the proton spectra of [2-^{15}N]creatine and [2/3-^{15}N]creatinine. Such line broadening is not apparent, however, from ^{31}P NMR spectra of the labeled phosphocreatinines and phosphocreatines.

The results of the ^{15}N and ^{1}H NMR studies reported here are consistent only with protonation exclusively at N-2 on creatinine at low pH. The evidence further suggests that the most important resonance form is that in which conjugation with the carbonyl moiety is present, although the other two forms are likely to make small contributions.

The ^{15}N and ^{31}P NMR data were used to establish unequivocally that the site of phosphorylation in phosphocreatinine is at N-2, i.e., on the exocyclic nitrogen. The phosphoryl group of phosphocreatinine thus has the opposite stereochemistry from that required for phosphoryl transfer to MgADP in the reaction catalyzed by creatine kinase. The fact that phosphocreatinine and creatinine are not substrates in the creatine kinase reaction has been interpreted to mean that the presence of a carboxyl group is necessary for substrate activity. The essential role of the carboxyl group is now called into question.

Finally, [2-^{15}N]phosphocreatine was found by this investigation to be potentially useful as a probe of the stereospecificity and kinetics of the creatine kinase reaction. Two prerequisites for this use were met. First, the ^{31}P-^{15}N one-bond coupling constant was found to be 18 Hz, not 3 Hz as reported by Brindle et al.[13] A J_{NP} value of 18 Hz is more than sufficient to allow resolution of ^{31}P NMR signals in spite of both the ^{15}N-induced chemical shift change and any line broadening induced by the ^{14}N quadrupolar nucleus. Therefore, the phosphorus resonances of [2-^{15}N]-phosphocreatine and [3-^{15}N]phosphocreatine can be resolved in order to quantitate their relative amounts in solution. Second, as stated above, the last step in the synthesis of [2-^{15}N]-phosphocreatine was not accompanied by scrambling of the label. This proves that specifically labeled phosphocreatine does not undergo ^{14}N/^{15}N positional isotope exchange all by itself.

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P_1 Residue Determines the Operation of the Catalytic Triad of Serine Proteases during Hydrolyses of Acyl-Enzymes

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Abstract: Proton inventories (rate measurements in mixtures of H_2O and D_2O) were determined for hydrolyses of acyl-enzymes that were formed during the reactions of porcine pancreatic elastase, human leukocyte elastase, and bovine pancreatic α-chymotrypsin with three peptidic substrates that differed in the P_1 amino acid residue (Ala, Val, or Phe). "Bowl-shaped" proton inventories were observed for substrates that fulfilled the enzyme's P_1 specificity requirements, while linear proton inventories were observed for P_1-nonspecific substrates. These results suggest that the P_1 residues of peptidic substrates play a critical role in determining whether the catalytic triad of serine proteases acts in a coupled manner with two-proton catalysis or simply operates as a one-proton catalyst.

The proton inventory technique[1] has been used to probe the operation of the catalytic triad[2] for a number of serine protease-catalyzed reactions.[3-9] In general, linear proton inventories, suggesting simple, one-proton catalysis by the active site histidine, have been found for minimal, nonspecific substrates, such as *p*-nitrophenyl acetate, while "bowl-shaped" proton inventories, consistent with two-proton catalysis and full functioning of the catalytic triad, have been found for specific substrates, such as tri- and tetrapeptide-based anilides and esters. The goal of these studies was to establish substrate structural requirements for the effective operation of the catalytic triad. Effective operation of the catalytic triad with substrate prolongation suggests a structural coupling[3] of this catalytic unit with remote protease subsites.[2] Interactions at remote subsites are known to play several important

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(1) Venkatasubban, K. S.; Schowen, R. L. *CRC Crit. Rev. Biochem.* **1985**, *17*, 1-44.
(2) Kraut, J. *Annu. Rev. Biochem.* **1977**, *46*, 331-358.
(3) Stein, R. L.; Strimpler, A. M.; Hori, H.; Powers, J. C. *Biochemistry* **1987**, *26*, 1305-1314.
(4) Stein, R. L. *J. Am. Chem. Soc.* **1983**, *105*, 5111-5116.
(5) Stein, R. L; Elrod, J. P.; Schowen, R. L. *J. Am. Chem. Soc.* **1983**, *105*, 2446-2452.
(6) Quinn, D. M.; Elrod, J. P.; Ardis, R.; Friesen, P.; Schowen, R. L. *J. Am. Chem. Soc.* **1980**, *102*, 5358-5365.
(7) Elrod, J. P.; Hogg, J. L.; Quinn, D. M.; Venkatasubban, K. S.; Schowen, R. L. *J. Am. Chem. Soc.* **1980**, *102*, 3917-3922.
(8) Hunkapiller, M. W.; Forgac, M. D.; Richards, J. H. *Biochemistry* **1976**, *15*, 5581-5588.

0002-7863/87/1509-4387$01.50/0