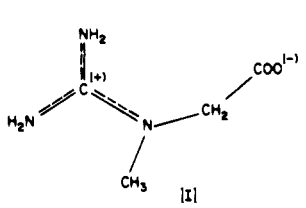
Stereospecificity of Creatine Kinase. Crystal Structure of 1-Carboxymethyl-2-imino-3-phosphonoimidazolidine[1]

Sir:

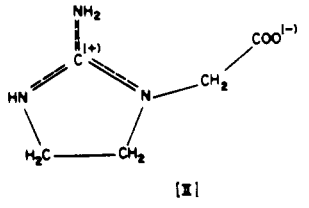
In the contraction–relaxation of muscle, adenosine 5′-triphosphate (ATP) is considered to be the immediate source of chemical energy, but a major reservoir of chemical energy is available as creatine phosphate. The formation of creatine phosphate is catalyzed by creatine kinase in the following reaction:

$$\text{creatine} + \text{MgATP}^{2-} \rightleftharpoons \text{creatine phosphate}^{2-} + \text{MgADP}^{-} + \text{H}^{+} \quad (1)$$

Kenyon and co-workers[2-3] have synthesized and studied a number of analogues of creatine (I); in particular 1-carboxymethyl-2-iminoimidazolidine (cyclocreatine) (II) has been



[I]



[II]

shown to be an excellent substrate for creatine kinase in vitro.[3] Griffiths and Walker have shown that dietary cyclocreatine is accumulated as the phosphorylated form in muscle, heart, and brain.[4] Furthermore, in vivo utilization of phosphocyclocreatine has recently been demonstrated in Ehrlich ascites tumor cells, indicating that this synthetic phosphagen can serve as a functional source of high energy phosphate.[5]

On the basis of NMR studies, the structure of the enzymatically phosphorylated cyclocreatine has been reported as 1-carboxymethyl-2-phosphonoiminoimidazolidine[2] and later as 1-carboxymethyl-2-imino-3-phosphonoimidazolidine;[6] the difference between these two compounds being the location of the phosphate moiety.

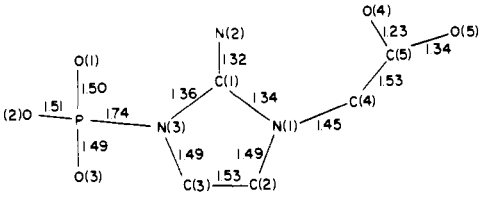
To unambiguously establish the site of phosphorylation, we have undertaken a single-crystal structure determination of the enzymatically active phosphorylation product of cyclocreatine. The determination unequivocally shows the product to be 1-carboxymethyl-2-imino-3-phosphonoimidazolidine (phosphocyclocreatine). The stereospecificity of creatine kinase is discussed in light of this finding.

Phosphocyclocreatine was chemically synthesized by Annesley and Walker[7] and demonstrated to be identical with the enzymatically active compound. Very large thin plate crystals could be grown (5 × 5 × 0.1 mm) from an aqueous solution containing 65% ethanol. One of these plates was trimmed to 0.5 × 0.3 × 0.1 mm for data collection. Since the crystalline compound is very hygroscopic, the crystal was mounted inside a glass microcapillary, evacuated, and sealed. Integrated intensities for 1931 independent reflections having d_{min} of 0.85 Å were measured on a Syntex $P2_1$ diffractometer with Nb-filtered Mo Kα radiation. To assess any possible crystal decay due to irradiation, the plane of data containing reflections of the type 0kl was calculated at the beginning and the end of the data collection. However, no deterioration was observed.

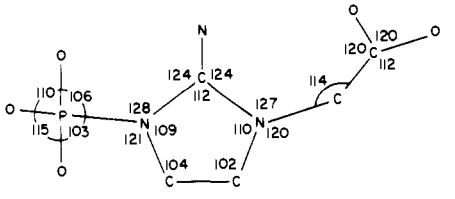
Elemental analysis of the crystalline compound showed the following stoichiometry: $P_1O_7N_3C_5Li_2H_{12}$. This is consistent with the hydrated structure, $Li_2PO_5N_3C_5H_8 \cdot 2H_2O$. Systematic extinctions revealed the space group to be either $P2/c$, or Pc, with four molecules in the unit cell (excluding solvent). The crystal lattice parameters are a = 22.22 (2), b = 5.54 (1), c = 9.444 (1) Å; β = 95.35 (15)°. The calculated density is 1.55 g/cm^3 and the density determined by flotation technique is 1.62 g/cm^3. A plot of the reflection intensities vs. sin θ/λ suggested that the structure was centrosymmetric. Therefore multisolution tangent formula (MULTAN)[8] was utilized in space group $P2/c$ to generate starting phases. The E map corresponding to the highest overall figure of merit revealed the phosphate group and the imidizolidine ring. A Fourier synthesis using phases calculated from these initial atoms showed a disordered carboxymethyl group. It further indicated that two carboxymethyl group orientations are not centrosymmetrically related; the true space group must therefore be Pc. Fourier maps calculated in space group Pc using phases derived from the two complete phosphocyclocreatine structures in the asymmetric unit showed the location of the oxygen atoms of water molecules and eventually lithium atoms. Some hydrogen atom positions have been determined by difference Fourier maps and others calculated.

Initial full-matrix least-squares refinements of all nonhydrogen atoms, using Hughes weighting scheme[9] and including isotropic temperature factors, resulted in an unweighted reliability index, R, of 12.8%. The positional and anisotropic thermal parameters of all nonhydrogen atoms were then refined using 1819 reflections for which the observed intensity was greater than three times the corresponding standard deviation. The water oxygen and lithium atoms were constrained to vibrate isotropically. The positional parameters for the hydrogen atoms were refined in the final cycles and the isotropic temperature factors were fixed to the value estimated from the Wilson plots. The final unweighted R was 8.3% and the weighted R was 3.5%.

The phosphate group is bonded to the ring nitrogen, N(3), establishing the structure of the active compound as 1-carboxymethyl-2-imino-3-phosphonoimidazolidine (III). The bond lengths (in angstroms) and angles (degrees) between nonhydrogen atoms, averaged over the two independent molecules in the asymmetric unit, are indicated in IIIa and IIIb,



[IIIa]



[IIIb]

respectively. (For clarity, hydrogen atoms were omitted in IIIa and IIIb.) The overall standard deviations as a result of averaging the two molecules are 0.3 Å for bond lengths and 2° for bond angles. The atomic coordinates for the two molecules in the asymmetric unit are given in Table I.

Least-squares planes were calculated through the four atoms N(1), C(1), N(2), and N(3) of the guanidino portion in the two independent molecules. The maximum displacement of any of the four atoms from the calculated plane in either molecule was 0.03 Å. Least-squares-planes calculation through the five atoms of the imidazolidine ring in both molecules showed that the rings themselves are planar, but that the phosphate and carboxymethyl groups are trans with respect to the ring plane (average displacement of 0.185 Å for the phosphorus atom and 0.15 Å for the C(4) atom of the carboxymethyl group). The three-dimensional packing arrangement and solvent structure are shown in Figure 1. The pseudocenter of symmetry is lo-

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Table I. Fractional Atomic Coordinates (× 10^3) and esd's for Nonhydrogen Atoms of Phosphocyclocreatine Molecules. Coordinates for Solvent Molecules (Li^+ and Water) Are Also Listed[a]

	X	Y	Z
C(1)	774 (1)	764 (3)	743 (1)
C(2)	745 (1)	454 (4)	586 (1)
C(3)	817 (1)	453 (3)	614 (2)
C(4)	664 (1)	677 (4)	692 (1)
C(5)	641 (1)	816 (4)	565 (2)
N(3)	827 (1)	681 (3)	696 (1)
N(2)	768 (1)	936 (3)	827 (2)
N(1)	729 (1)	617 (3)	698 (2)
O(1)	932 (1)	780 (2)	637 (1)
O(2)	921 (1)	569 (2)	858 (1)
O(3)	888 (1)	1001 (2)	835 (1)
O(4)	675 (1)	919 (2)	488 (1)
O(5)	588 (1)	939 (4)	569 (2)
P	898 (1)	768 (1)	764 (1)
C(1A)	−773 (1)	−757 (3)	−742 (2)
C(2A)	−749 (1)	−452 (3)	−582 (2)
C(3A)	−813 (1)	−476 (3)	−609 (2)
C(4A)	−665 (1)	−685 (4)	−693 (2)
C(5A)	−639 (1)	−851 (5)	−568 (2)
N(3A)	−825 (1)	−694 (2)	−697 (1)
N(2A)	−770 (1)	−954 (2)	−831 (1)
N(1A)	−726 (1)	−630 (4)	−686 (2)
O(1A)	−935 (1)	−779 (2)	−635 (1)
O(2A)	−920 (1)	−561 (1)	−861 (1)
O(3A)	−889 (1)	−1002 (1)	−838 (1)
O(4A)	−672 (1)	−906 (3)	−479 (1)
O(5A)	−583 (1)	−795 (3)	−549 (1)
P(A)	−898 (1)	−769 (1)	−762 (1)
Li(1)	933 (1)	757 (3)	438 (2)
Li(2)	−935 (1)	−751 (5)	−426 (3)
Li(3)	−001 (2)	497 (3)	748 (3)
Li(4)	475 (1)	592 (5)	157 (3)
O(W1)	979 (1)	755 (1)	100 (1)
O(W2)	−980 (1)	−760 (2)	−103 (1)
O(W3)	553 (1)	678 (3)	893 (2)
O(W4)	473 (1)	001 (3)	693 (2)

[a] The additional designation "A" is for the other molecule in the asymmetric unit and designation "W" for the oxygen atoms of the water molecules.

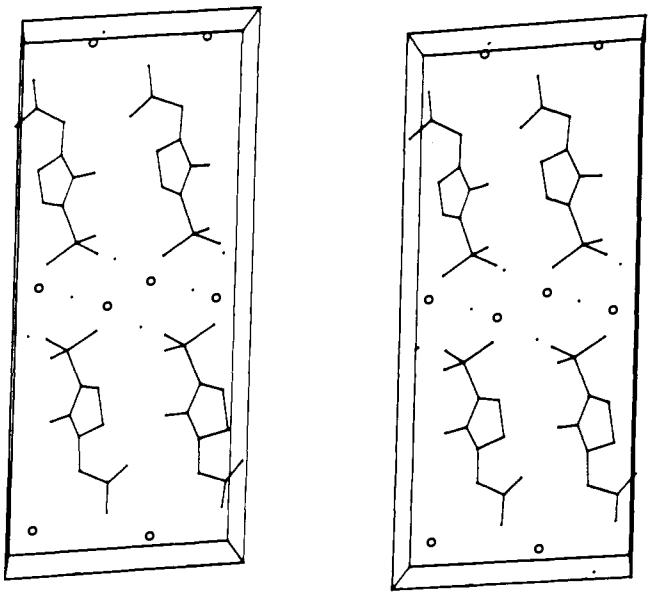

Figure 1. A stereodrawing of the crystal packing of phosphocyclocreatine. The nonbonded solvent atoms are represented as follows: water oxygens, large open circles; Li^+, small filled circles. The unit cell is viewed down the crystallographic x axis.

cated at the center of the cell. The lack of true centrosymmetry is most evident in the region of the carboxymethyl groups.

It is interesting to note that the bond distances and angles of the common structural element of phosphocyclocreatine (this study), phosphocreatine,[10] and creatine[11-12] are not significantly different. Additionally, the phosphocreatine portion in the phosphocyclocreatine compound is similar in conformation to that found in phosphocreatine. This configuration avoids unfavorable steric and electrostatic repulsion between the charged COO^- and PO_3^{2-}.

In reaction 1, which is catalyzed by creatine kinase, phosphorylation of creatine (I) could occur on the free nitrogen either cis or trans to the methyl group. The addition of methylene bridge to form the cyclocreatine II fixes the stereochemistry of the two possible sites of phosphorylation. Since analysis of the enzymatically synthesized phosphocyclocreatine showed the formation of only one isomer,[1,7] establishment of the structure of the active isomer as a 3-phosphono compound III demonstrates unequivocally that creatine kinase catalyzes the stereospecific phosphorylation of creatine at the nitrogen group which is cis to the methyl group. This study further indicates that the positions and angles of the atoms fixed by the ring structure in II are very close to those adopted by creatine in the enzyme-substrate complex.

References and Notes

(1) Supported in part by Grant C-581 from the Robert A. Welch Foundation and Grant GM-21371 from the National Institutes of Health.
(2) G. L. Rowley, A. L. Greenleaf, and G. L. Kenyon, *J. Am. Chem. Soc.*, (1971).
(3) A. C. McLaughlin, M. Cohn, and G. L. Kenyon, *J. Biol. Chem.* **247**, 4382-4388 (1972).
(4) G. R. Griffiths and J. B. Walker, *J. Biol. Chem.*, **251**, 2049-2054 (1976).
(5) T. M. Annesley and J. B. Walker, *J. Biol. Chem.*, **253**, 8120-8125 (1978).
(6) G. E. Struve, C. Gazzola, and G. L. Kenyon, *J. Org. Chem.*, **42**, 4035 (1977).
(7) T. M. Annesley and J. B. Walker, *Biochem. Biophys. Res. Commun.*, **74**, 185-190 (1977).
(8) C. Germain, P. Main, and M. M. Woolfson, *Acta Crystallogr., Sect. A*, **27**, 368-376 (1971).
(9) E. W. Hughes, *J. Am. Chem. Soc.*, **63**, 1737-1752 (1941).
(10) J. R. Herriott and W. E. Love, *Acta Crystallogr., Sect. B*, **24**, 1014-1027 (1968).
(11) H. Mendel and D. C. Hodgkin, *Acta Crystallogr.*, **7**, 443-446 (1954).
(12) L. H. Jensen, *Acta Crystallogr.*, **8**, 237-240 (1955).
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G. N. Phillips, Jr., J. W. Thomas, Jr.
T. M. Annesley,[13] F. A. Quiocho*
Department of Biochemistry, Rice University
Houston, Texas 77001

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Formation of 1-Ethylxanthyl-2,7-diaminomitosene and 1,10-Diethylxanthyl-2,7-diaminodecarbamoylmitosene in Aqueous Solution upon Reduction-Reoxidation of Mitomycin C in the Presence of Potassium Ethylxanthate

Sir:

Mitomycin C is a clinically useful antitumor agent which is produced by several species of *Streptomyces*.[1] Iyer and Szybalski and others have demonstrated that mitomycin C cross links DNA in vivo and, after reduction by sodium dithionite in aqueous buffers, in vitro. An activation mechanism for mitomycin C was proposed by Iyer and Szybalski[2] and by Patrick et al.[3] which was largely based on the acid-catalyzed chemistry of mitomycin C.[4] It was suggested that two electrophilic sites would be generated at positions 1 and 10 in mitomycin C, after loss of the 9a-methoxy substituent, opening

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