Phosphorus Nuclear-Magnetic-Resonance Studies of a Transition-State Analogue Complex of Creatine Kinase

By E. JAMES MILNER-WHITE* and DAVID S. RYCROFT†

*Department of Biochemistry and †Department of Chemistry, University of Glasgow,
Glasgow G12 8QQ, Scotland, U.K.

(Received 8 August 1977)

³¹P nuclear-magnetic-resonance spectra of MgADP bound to creatine kinase in the presence of creatine and NO₃[−] ions show that there are two non-identical forms of the bound nucleotide. The sites have different affinities for the nucleotide. MgADP at the high-affinity site is in slow exchange ($k < 125\text{ s}^{-1}$ at 27°C) with free MgADP.

The nitrate ion in the creatine–NO₃[−]–MgADP–creatine kinase complex appears to mimic the phosphoryl group that is transferred between creatine and MgADP in the working enzyme; because the complex binds tightly to the enzyme, it is considered to be an analogue of the transition state or of another intermediate state of the reaction (Milner-White & Watts, 1971). McLaughlin (1974) and Price & Hunter (1976) have indicated that, in the presence of creatine and NO₃[−] ions, there is a different affinity for binding of MgADP to two sites present in equal amounts in the enzyme population. Price & Hunter (1976) also observed differential chemical reactivity of the essential thiol groups of the enzyme in the presence of the same substrate and NO₃[−] combination. These findings were interpreted by these authors in terms of interaction within the dimer between subunits with identical amino acid sequences. However, Williamson *et al.* (1977) have shown that purified and native creatine kinase are composed of two peptide chains, of slightly different molecular weights, present in roughly equal amounts; thus it may not be necessary to invoke subunit interactions to explain the findings of Price & Hunter (1976). In the present work we have been studying the binding of substrates to creatine kinase by using ³¹P n.m.r. (nuclear magnetic resonance), a technique used successfully with the monomeric arginine kinase (Rao *et al.*, 1976; Rao & Cohn, 1977).

Experimental

The preparation and storage of rabbit skeletal muscle creatine kinase, the measurement of its specific activity and the chemicals used were all as described by Milner-White & Kelly (1976). The enzyme was concentrated at 0°C with Centriflo membrane cones (Amicon Ltd., High Wycombe, Bucks., U.K.), followed by vacuum dialysis at 0°C by using Ultra-thimbles UH 100 (Schleicher und Schüll, Dassel, Germany). Enzyme concentrations as high as 5 mM were prepared and were stored in Bicine [N,N-bis-(2-hydroxyethyl)glycine; 10 mM], adjusted with NaOH to pH 9.0, for up to 3 days at 0°C.

Proton-noise-decoupled ³¹P n.m.r. spectra were obtained at 40.5 MHz in the Fourier transform mode, by using a Varian XL-100-12 n.m.r. spectrometer with a VFT-100 accessory and a variable temperature probe, from H₂O solutions (0.9 ml) contained in spinning 10 mm (outer diameter) tubes. The ²H field/frequency lock operated on the resonance of C₆D₆ contained in an inner concentric tube. Chemical shifts are expressed as positive to low field of external 85% (w/w) H₃PO₄. The normal spectral-acquisition conditions were: spectral width, 2500 Hz; pulse angle, ~50°; acquisition time, 0.8 s; exponential weighting factor, −0.2 s; 8K Fourier transform, 0.6 Hz per data-point in the transformed spectrum. Enzyme activities measured after overnight experiments at 27°C were found to be unchanged.

Results and Discussion

Fig. 1(a) shows the ³¹P n.m.r. spectrum of MgADP at pH 9.0 and 27°C; the lower-field doublet arises from the β-phosphorus (β-P) atom. When the MgADP is bound to creatine kinase as the MgADP–creatine complex, the β-P signal, shown in Fig. 1(b), is broadened and shifted downfield by 1.8 p.p.m.; bound and free MgADP are in fast exchange (on the n.m.r. time scale), as was shown by observing one signal at an intermediate shift on adding excess MgADP.

In the presence of excess nitrate and creatine, and with an equimolar ratio of enzyme and MgADP (i.e. a 2:1 ratio of active sites to MgADP), the β-P signal shifted upfield by 3.1 p.p.m., giving a broad signal, seen in Fig. 1(c); the spectrum in Fig. 1(d) shows that when the MgADP concentration was doubled a new broad β-P signal appeared, 1.4 p.p.m. to lower field of the original β-P signal, which remained unchanged. Fig. 1(e) shows the spectrum of the same solution at 4°C; here the downfield signal has sharpened into a doublet, whereas the upfield signal is unchanged. Addition of excess MgADP at 27°C as seen in Fig. 1(f) increased the intensity of the downfield β-P signal and the sharpness, so that the doublet structure was visible. In all these spectra the signals from the

α -phosphorus atom showed only small changes, consistent with those from the β -P signals, but less clear because of overlap.

Since the chemical shift of the β -P atom of free MgADP and that of the downfield β -P signal of the added MgADP in Figs. 1(d) and 1(e) are almost identical, it is important to establish whether this signal corresponds to bound MgADP. The equilibrium-dialysis experiments of Price & Hunter (1976) at 18°C indicate that each enzyme molecule binds two molecules of MgADP strongly (K_D values of 1.5 μ M and 18 μ M) in the presence of creatine and NO_3^- ions, a result that we have confirmed at 4°C (E. J. Milner-White & D. S. Rycroft, unpublished work). Under the conditions of our n.m.r. experiment, 20% of the ADP not bound to the enzyme is unchelated to Mg^{2+} ions ($K = 4100 \text{ M}^{-1}$ at 27°C; Phillips *et al.*, 1966). Since the binding of ADP as the creatine- NO_3^- -ADP complex has one-half of the affinity to the enzyme of the Mg^{2+} -containing complex (Price & Hunter, 1976), only 9% of the bound complexes are expected to lack Mg^{2+} ions. At 4°C the corresponding value is 6.5%.

The presence of one β -P signal in Fig. 1(c) and two in similar proportion in Fig. 1(d) appears to indicate that the two subunits of creatine kinase bind MgADP with different affinities in the presence of creatine and NO_3^- ions as proposed by Price & Hunter (1976). In this case the two chemically shifted β -P signals also suggest asymmetry in the dimeric enzyme fully bound to the transition-state analogue complex. An alternative explanation might be that two different types of enzyme molecule with different affinities for the complex are present; the heterogeneity of the enzyme found by Williamson *et al.* (1977) may support this idea.

Comparison between Figs. 1(d) and 1(e) shows that the downfield β -P signal sharpens at low temperatures and is therefore in the slow-exchange region. The exchange process presumably also involves the upfield

Fig. 1. ^{31}P n.m.r. spectra of enzyme-bound and free MgADP. Samples contained creatine (30 mM), Bicine (25 mM) adjusted to pH 9.0 with NaOH, Mg^{2+} ions added as the acetate salt to a concentration of 1 mM above that of the ADP, and: (a) ADP, 2 mM; at 27°C (10 K pulses); (b) ADP, 6 mM; creatine kinase, 3.10 mM; at 27°C (60 K pulses); (c) ADP, 3.11 mM; creatine kinase, 3.12 mM; NaNO_3 , 0.1 M; at 27°C (43 K pulses); (d) ADP, 6.44 mM; creatine kinase, 3.12 mM; NaNO_3 , 0.1 M; at 27°C (55 K pulses); (e) the same solution as (d) at 4°C (31 K pulses); (f) ADP, 9.33 mM; creatine kinase, 3.25 mM; NaNO_3 , 0.1 M; at 27°C (24 K pulses). Creatine kinase concentrations are expressed as the molarity of the dimeric enzyme (mol.wt. = 82100). It should be noted that the intensity scale is not the same for all the experiments.

β -P signal; possible reasons why this signal is not sharpened at 4°C are the onset of exchange broadening due to a conformational process, or lower mobility of the more tightly bound molecule of MgADP.

The rate constants for exchange cannot be calculated exactly, since not all contributions to the line-width are known. However, as the signals are not coalesced, it can be stated that the first-order rate constants for exchange of MgADP both between the two MgADP-binding sites in the presence of creatine and NO_3^- ions, and between free MgADP and the strongly bound MgADP site in the creatine- NO_3^- -MgADP complex, must be less than 125 s^{-1} at 27°C. Since free MgADP and the more weakly bound MgADP in the presence of creatine and NO_3^- ions show signals with similar chemical shifts, the exchange rates between them cannot be measured in the present work. Other evidence for slow conformational processes with various enzymes on binding transition-state analogues has been reviewed by Wolfenden (1976).

Double-resonance nuclear-Overhauser-effect experiments performed by James (1976) have provided evidence to show that rapid exchange (with respect to relaxation rates) occurs between free and bound MgADP in the presence of creatine and NO_3^- ions. However, the time scales of relaxation rates and chemical shifts are not the same; in addition, the solution composition used by James ($\text{NO}_3^-/\text{active sites} = 1:2$) is different from ours (large excess of NO_3^- ions over active sites).

The chemical shifts of the β -P signals of MgADP in Fig. 1 are similar to those reported by Rao & Cohn (1977) for the corresponding transition-state analogue complex of arginine kinase. They have proposed an interpretation of the chemical shifts ob-

served in terms of the chemical structures of the bound nucleotides. They also observed indications of doubling of the β -P resonance of MgADP, both in the presence and absence of arginine and NO_3^- ions, and suggested that this might arise from conformational heterogeneity; they stated that they were unable to obtain a reproducible set of line shapes. For creatine kinase we observe doubling of the β -P resonances of MgADP only for the transition-state analogue complex (Figs. 1*d* and 1*e*); our results indicate the possibility of intersubunit effects, but further work is needed to establish the nature of the signals observed.

We thank the Science Research Council for financial help towards purchase of the n.m.r. spectrometer.

References

- James, T. L. (1976) *Biochemistry* **15**, 4724–4730
- McLaughlin, A. C. (1974) *J. Biol. Chem.* **249**, 1445–1452
- Milner-White, E. J. & Kelly, I. D. (1976) *Biochem. J.* **157**, 23–31
- Milner-White, E. J. & Watts, D. C. (1971) *Biochem. J.* **122**, 727–740
- Phillips, R. C., George, P. & Rutman, R. J. (1966) *J. Am. Chem. Soc.* **88**, 2631–2640
- Price, N. C. & Hunter, M. G. (1976) *Biochim. Biophys. Acta* **445**, 364–376
- Rao, B. D. N. & Cohn, M. (1977) *J. Biol. Chem.* **252**, 3344–3350
- Rao, B. D. N., Buttlair, D. H. & Cohn, M. (1976) *J. Biol. Chem.* **251**, 6981–6986
- Williamson, J., Greene, J., Chérif, S. & Milner-White, E. J. (1977) *Biochem. J.* **167**, 731–737
- Wolfenden, R. (1976) *Annu. Rev. Biophys. Bioeng.* **5**, 271–306