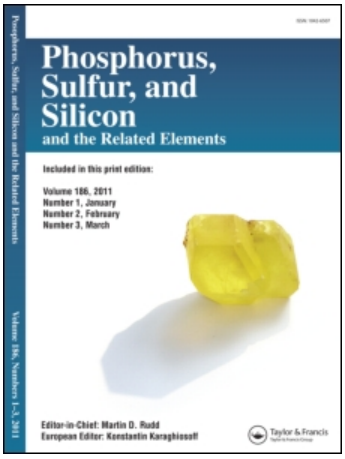


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# How Far Does the Itinerant Phosphoryl Group Move on a Phosphoryl-Transfer Enzyme?

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A summary of recent and ongoing attempts to characterize the conformations of reaction complexes bound at the active sites of ATP-utilizing enzymes by high-resolution NMR methods is presented.

*Keywords:* ATP-utilizing enzymes; enzyme mechanisms; enzyme-substrate interactions; cation activation

A number of biochemical pathways contain enzymes for which ATP is a substrate. Aside from ATP-ases, these enzymes fall into three categories, viz. phosphoryl transfer, adenyl transfer, and pyrophosphoryl transfer enzymes, on the basis of the transferred moiety from ATP and in the order of their abundance. Irrespective of the point of cleavage along the phosphate chain, all these enzymes require, for their catalysis *in vitro*, the presence of Mg(II) in the reaction complex. Structure-based elucidation of the mechanism of these enzymes has been sought for quite some time by standard macromolecular structure methodologies such as x-ray crystallography and Nuclear Magnetic Resonance (NMR). X-ray crystal structures of some of these proteins have provided the most reliable information on the active-site topography and the location of the substrate binding sites. The conformations deduced for nucleotides and nucleotide analogs cocrystallized with the proteins, however, have not been entirely satisfactory. For example, the location of the cation with respect to the nucleotide has not always been unequivocal. These anomalies are attributable to features of the crystallization process such as the effect of anions (e.g., sulfate or phosphate), and crystal packing effects that might trap the substrate in an unproductive conformation. While the NMR methods have not been applicable for protein structure determinations for most of these enzymes, which are of masses larger than 40 kD, they are best suited to determine the conformations of substrates bound to the enzymes for a number of reasons. 1. The experiments can be performed in liquid state, with the

substrates exclusively bound to the enzymes in a productive conformation. 2. The obligatory cation, Mg(II), may be substituted by activating paramagnetic cations such as Mn(II) and Co(II), and the distance-dependent paramagnetic effect on the nuclear spin relaxation of substrate nuclei in their vicinity (such as $^{31}P$, $^{13}C$, and $^{15}N$) can be used to reliably determine the substrate conformations. The location of the cation is thus unequivocal. 3. The interproton distances in the substrates can be determined by TRNOESY (transferred nuclear Overhauser effect spectroscopy) experiments. 4. Finally, the structure measurements can be made on enzyme-bound equilibrium mixtures as the substrates and products interconvert on the surface of the enzyme.

The basis for the two methods viz., relaxation effects due to paramagnetic cations and TRNOESY is straightforward. In nuclear relaxation measurements in the presence of paramagnetic cations, in which exchange of the cation between diamagnetic and paramagnetic complexes affects the observed relaxation rates, the relaxation rates will be structurally relevant only if the nuclear spin-lattice relaxation time in the paramagnetic complex ($T_{1M}$) is larger than the lifetime ($\tau_M$) of the complex i.e., $T_{1M} >> \tau_M$. Incorrect assessment of the role of this exchange was the primary flaw in previously published distance data from this method[1]. This problem was overcome by using the following procedure[2, 3]: 1. The measurements were made exclusively on enzyme-bound substrate complexes. 2. The relaxation measurements were made at three different frequencies and as a function of temperature to assess the role of exchange; $T_{1M}$ depends on frequency whereas $\tau_M$ does not, and the activation energies of $T_{1M}$ and $\tau_M$ are respectively in the ranges of 1-3 kcal/mole and over 5 kcal/mole. We used this protocol in the 80's for making $^{31}P$ relaxation measurements in various nucleotide complexes of creatine kinase[2], 3-P-glycerate kinase[4], arginine kinase[5], and adenylate kinase[6] using Mn(II) and Co(II) as the activating cations. The results showed that $\tau_M > T_{1M}$ in the presence of Mn(II) for cation-nucleotide distances up to about 6.5 Å. To determine shorter distances, Co(II), which induces weaker nuclear relaxation than Mn(II) (at the same distance), should be used[2, 3]. The cation is found to be directly chelated to all the phosphate groups of both E•CoATP and E•CoADP complexes of creatine kinase, 3-P-glycerate kinase, and arginine kinase with Co(II)-$^{31}P$ distances of about 3.0±0.2 Å[2-5]. In the case of the ATP complexes of adenylate kinase, however, the Co(II)-α-P distance is about 4.0 Å indicative of no direct coordination of this phosphate group[6].

In TRNOESY experiments published prior to 1993, typical sample protocols contained 1 mM enzyme sites and 10 mM nucleotide, and the NOE data was flawed due to weak nonspecific binding of the nucleotide with the protein[7, 8]. This point was strikingly demonstrated by TRNOESY experiments on samples in which ~10 mM MgADP was added to proteins (~1 mM) such as γ-globulin, and bovine serum albumin, which are not known to have specific nucleotide binding sites. The NOE patterns for adenosine protons in these samples closely resembled those obtained for creatine kinase with the same protocol[9]. A careful investigation of the dependence of the NOE on nucleotide concentration showed that adventitious binding begins to occur for nucleotide concentrations in excess of 2-3 mM. It is important to make a deliberate determination of this effect in order to choose sample protocols that will yield structurally relevant TRNOESY data. We performed such measurements to determine interproton distances in the adenosine moieties of nucleotides bound to four phosphoryl transfer enzymes, creatine kinase[9], arginine kinase[10], pyruvate kinase[11], adenylate kinase[12], one adenyl transfer enzyme, methionyl tRNA synthetase[13], and one pyrophosphoryl transfer enzyme, phosphoribosyl pyrophosphate synthetase[14]. Energy-minimized structures compatible with the distance data show that the glycosidic torsion angle for all the bound nucleotides fall in a narrow range of 50±7°. This similarity suggests a recognition and binding motif for the adenosine moiety at the active site of ATP-utilizing enzymes irrespective of the transferable group from ATP. The ribose pucker in the different enzyme-nucleotide complexes did not, however, show such a similarity.



Having thus determined the location of the cation with respect to the phosphate chain, and the conformation of the adenosine moiety, the final step in the characterization of the nucleotide requires a determination of the orientation of the cation-bound phosphate chain with respect to the adenosine[15]. We are currently making these measurements by utilizing [ul-$^{13}$C]-nucleotides, and Mn(II) as the activating cation.

We have recently begun making paramagnetic relaxation measurements on enzyme-bound equilibrium mixtures in order to probe the structural alterations in the reaction complex accompanying the enzyme turnover. The data were analyzed on the basis of a recently developed theory which treats the complications arising from the fact that there is an additional exchange process due to the interconversion of the reactants and products on the enzyme[16]. These measurements lead to the question posed in the title of this paper: "How Far Does the Itinerant Phosphoryl Group Move on a

Phosphoryl-Transfer Enzyme?". It is found that the Co(II)-γ-P(ATP) distance of 3.0 Å in E•CoATP•creatine complexes changes to a Co(II)-P-creatine distance of about 4.5 Å in the E•CoADP•P-creatine complex. The itinerant phosphoryl group thus moves at least 1.5 Å as the enzyme turns over. We do not know of any structural methodology, other than NMR, to determine dynamic structural information of this kind. Details of this work will be published soon.

Detailed structural characterization of the reaction complexes, as described above, coupled with crystallographic data on the amino acid environment at the active sites of proteins holds considerable promise for providing significant insights into the catalytic mechanisms operating in ATP-utilizing enzymes.

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