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[$^{18}O/^{16}O$]^{31}P-NMR Studies of Phosphoryl Transfer Enzymes

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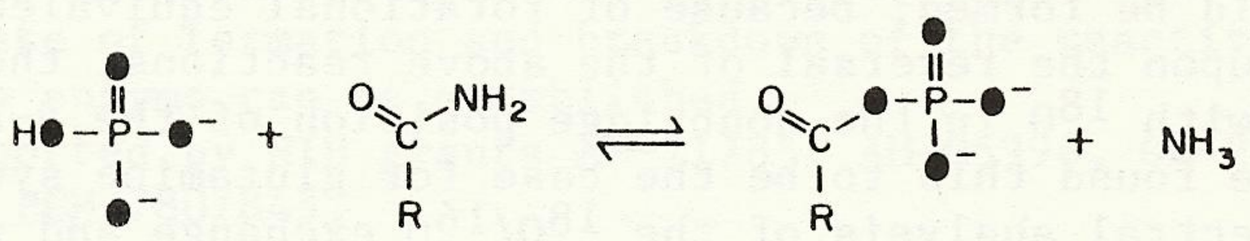
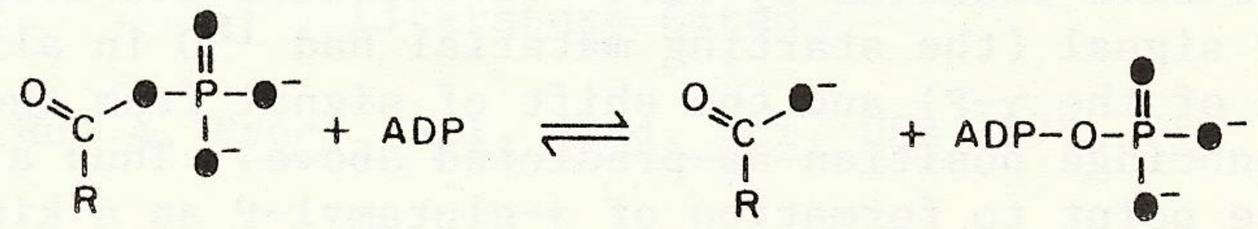
In 1978 Cohn and Hu (1) demonstrated the isotopic effect by ^{18}O on the ^{31}P-nmr spectrum of inorganic phosphate. For each ^{18}O that replaces an ^{16}O, an upfield shift of 0.021 ppm results. Thus the chemical shift for $HP^{16}O_4^=$ is 0.084 ppm downfield from $HP^{18}O_4^=$. Based on this observation it is obvious that any chemical event that potentially involves substitution or exchange of an ^{18}O for ^{16}O in phosphorus containing compounds can be followed by monitoring the ^{31}P-nmr spectrum throughout the course of the reaction. This article describes experiments from my laboratory on two enzymes that catalyze phosphoryl transfer reactions and our use of the [$^{18}O/^{16}O$]^{31}P-nmr methodology to detect intermediates in the enzymic reactions.

Glutamine synthetase catalyzes the formation of glutamine from ATP, gluatmate, and ammonia. The other products are ADP and P_i. The reaction mechanism is though to proceed through a γ-glutamyl phosphate intermediate. We have shown that incubation of ADP, glutamine, and [^{18}O]P_i with glutamine synthetase resulted in the loss of ^{18}O from the P_i (2). Analysis of the data showed that only one ^{18}O was lost per encounter and that the rate constant for exchange was 5-7 times faster than net turnover of products. This was also demonstrated by Stokes and Boyer (3) using mass spectrometry.



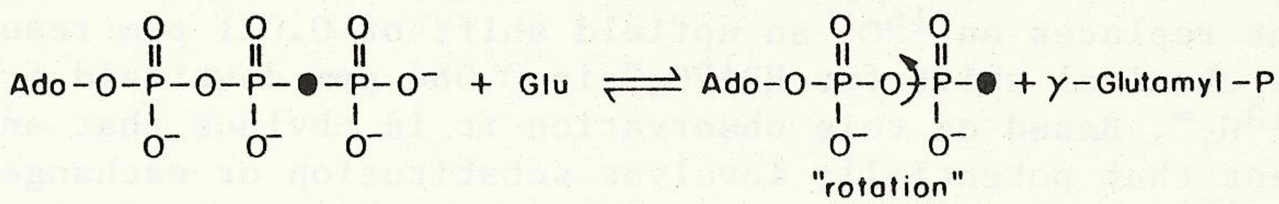
(I)

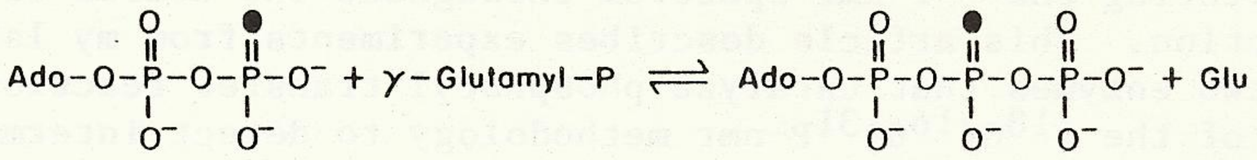


(I) (II)

This exchange reaction is explained by assuming that the above reactions are occurring on the enzyme surface and that the γ-carboxyl of glutamate (II) is free to rotate, thus allowing, upon reversal of the reaction, the formation of γ-glutamyl phosphate (I) with an ^{16}O in the bridge position. Reaction of I with NH_3 would then produce P_i with only 3 atoms of ^{18}O instead of the original 4. Since a random distribution of ^{18}O is maintained at all times, only one ^{18}O is lost per encounter with the enzyme and thus P_i must be dissociating from the enzyme faster than glutamine.

Recently Midelfort and Rose (4) introduced a positional isotope exchange technique that is also suitable for study by the ^{18}O chemical shift technique. Briefly, this technique follows the exchange of label from one part of a substrate to another due to rotational equivalence of some intermediate. The method was first applied to glutamine synthetase in the reaction:





ATP is synthesized with ^{18}O in the β-γ bridge position. In phosphate-containing species such as ATP where the phosphorus (α, β, and γ) and oxygen (bridge and nonbridge) atoms are in different environments the effect of ^{18}O substitution on the ^{31}P chemical shift is dependent on the oxygen environment. The shift is largest for those oxygens with most double bond character. For the β-P, the shift per ^{18}O atom is 0.016 ppm for the β-γ bridge position and 0.028 ppm for the β nonbridge position.

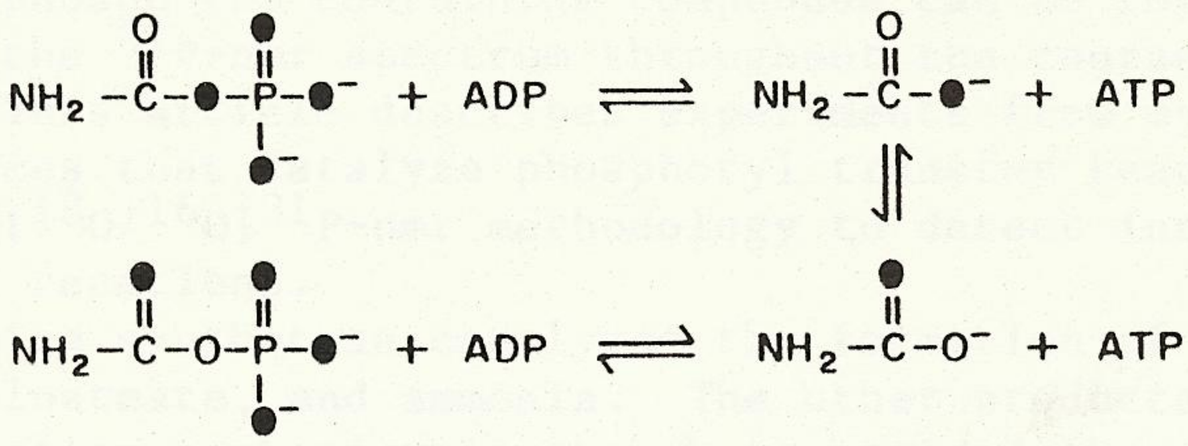
Presuming glutamine synthetase catalyzed the formation of γ-glutamyl-P from ATP and glutamate in the absence of NH_3, then ADP would be formed; because of rotational equivalence it would allow, upon the reversal of the above reactions, the formation of ATP with ^{18}O in the nonbridge position of the β-P. Midelfort and Rose found this to be the case for glutamine synthetase by mass spectral analysis of the $^{18}O/^{16}O$ exchange and we have followed this reaction by nmr. We detected the loss of ^{18}O from the γ-P signal (the starting material had ^{18}O in all four oxygens of the γ-P) and the shift of signal from the β-γ bridge to β nonbridge position as predicted above. Thus all lines of evidence point to formation of γ-glutamyl-P as a kinetically competent intermediate in catalysis.

Carbamyl phosphate synthetase catalyzes the synthesis of carbamyl-P from HCO_3^-, glutamine, and 2 moles of ATP. The enzyme also catalyzes the HCO_3^--dependent hydrolysis of ATP. Raushel and Villafranca (5) followed the exchange of ^{18}O from the bridge to the nonbridge position of [γ-^{18}O]ATP after incubation with enzyme and bicarbonate. The exchange rate was 0.4 times the rate of ADP formation. These results support the formation of carboxy phosphate as the first intermediate in the catalytic sequence. The rate of formation of intermediates in the reaction was also studied using rapid reaction techniques by Raushel and Villafranca (6) and the data agree with the ^{31}P-nmr studies presented above.

The positional isotope exchange has also been measured with ^{31}P-nmr in the reverse reaction of carbamyl phosphate synthetase:

$$\text{Carbamyl-P} + \text{ADP} \rightarrow \text{ATP} + NH_3 + HCO_3^-.$$

Carbamyl phosphate was synthesized with ^{18}O in all oxygens except the carbonyl oxygen of carbon. The exchange of the bridge oxygen into the carbonyl oxygen was followed by nmr and was evidence for the following series of reactions



These data support the formation of a second intermediate in the reaction pathway, viz, carbamate ($NH_2CO_2^-$) and its formation is ~4 times faster than ATP formation for the reverse reaction outlined above.

In conclusion the [$^{18}O/^{16}O$]^{31}P-nmr method is a powerful technique for the study of reaction intermediates in phosphoryl transfer reactions. Both the nature of the reactive species as well as the rate of formation and breakdown of the reactive species on the enzyme can be established.

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