EVIDENCE FOR AN INTERMEDIATE IN MYOSIN HYDROLYSIS*

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...e hydrolysis of ATP by myosin was discovered the mechanism of this reaction has been a subject of intensive research. In some way the energy of the phosphate bond must be transferred to muscle protein so that chemical energy can be transformed into mechanical work. Formation of a phosphoryl-myosin intermediate has been suggested by many workers but evidence for such an intermediate has been elusive. ADP^{32}-ATP exchanges gave negative results(1) and no P^{32}-labeled myosin, other than that absorbed by non-covalent forces(2), has yet been identified with the hydrolysis reaction.

If one examines the possible ways in which the ATP molecule might split, two possibilities exist. Cleavage could occur at the terminal phosphorus or at the middle phosphorus atom. To resolve this problem, O^{18} studies were initiated. Under all circumstances cleavage occurred between the terminal phosphorus and its oxygen atom (cf. Table 1) as shown by the absence of O^{18} in the ADP produced.(3,4) Under certain circumstances, e.g. in the presence of calcium ion, the amount of

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oxygen observed in the terminal phosphorus introduced by O^{18} from the water was the theoretical number, i.e. one out of the four of the phosphate ion.(1) However, in other cases, e.g. in the presence of magnesium ion, this number was found to be much higher than theoretical, for example, three out of the four oxygen atoms.(4) The extra oxygens must have been introduced by an exchange reaction.

In studying the nature of this exchange reaction, it was soon found that the results could be explained by postulating an intermediate step in the myosin catalyzed hydrolysis.(4) Myosin did not catalyze exchange of oxygen between H_2O and P_i nor did it cause exchange between H_2O and unhydrolyzed ATP. Since exchange did not occur with the initial reactant, ATP, nor the final product, P_i, we deduced that it occurred at some intermediate stage in the reaction. Although the precise nature of the intermediate was not delineated from this experiment, it was the first direct evidence for an intermediate stage in myosin hydrolysis and the O^{18} was used as a probe for determining some of the properties of this intermediate.

A year or so ago Dr. Boyer was examining this system from a different angle and observed that there was a lack of rigor in the reasoning leading to an intermediate stage. To test this he and Dr. Dempsey(5) incubated O^{18}-labeled P_i in a medium of H_2O^{16}. When they did this an exchange between the O^{18} of the P_i and the O^{16} of the water occurred during the hydrolysis of ATP. In the absence of ATP, this exchange was not detectable. Thus, Boyer and Dempsey confirmed our findings that no exchange occurred with P_i of the medium

in the absence of ATP but they went further and established that in the presence of ATP an exchange with the P_i did occur. They then performed kinetic analyses which indicated that all of the exchange occurring in myosin hydrolysis could be accounted for by this ATP induced exchange with the P_i of the medium. If true, this would essentially eliminate the evidence for the intermediate stage that we had postulated.

To clarify this discrepancy, we performed further experiments(6) of the types reported in Tables 2 and 3. Parallel experiments were set up in which the conditions were identical except for the initial position of the O^{18} label. The parallel experiments used the same buffer, magnesium chloride, and initial amounts of P_i and ATP. In one case, the water contained O^{18} and the P_i was unlabeled whereas in the other case the P_i contained O^{18} and the water was unlabeled. The latter measures exchange occurring only via P_i of the medium. The former measures exchange occurring both at the medium level and at the intermediate stage. If the two values were the same, the evidence for an intermediate stage would be excluded. If the latter were lower than the former, evidence for such an intermediate stage would be established. In the P_iO^{18} experiments shown in Table 2 it can be seen that approximately 0.5 oxygen atoms were exchanged per phosphate group during ATP hydrolysis. In the parallel experiment with O^{18} initially in the water the P_i of the final product contains approximately 1.4 atoms per phosphate ion. This is an average value for the total P_i present at the end of the experiment, i.e. P_i produced by hydrolysis and P_i initially present. Thus, many more

atoms are exchanged in the experiment which is sensitive to an intermediate as well as a medium exchange than in an experiment which is sensitive only to a medium exchange. Thus, both types of exchange must exist.

If one assumes that the exchange is made up of two components, an exchange reaction with the P_i of the medium as postulated by Boyer and another exchange occurring at the intermediate level as originally postulated by us, it is possible to correct these values so that one may calculate the number of atoms exchanged at the intermediate stage. When this is done it is found that two atoms of oxygen exchanged per phosphate group at the intermediate stage (last column of Table 2). It should be emphasized that the two atoms introduced by exchange are over and above the one atom introduced due to cleavage of the phosphorus-oxygen bond by hydrolysis. The figure 2.0 for intermediate exchange is greater than the 1.4 observed for total P_i because the latter is averaged over all the phosphate present including the 0.005 M P_i added initially. The 2.0 figure is calculated P_i for the P_i produced by hydrolysis since the initially added P_i should undergo only a medium exchange.

Although the difference between the exchange values in the ATP experiments were outside of experimental error, it was still desirable to establish this conclusion with even greater certainty. If the medium exchange was a time-dependent exchange, as pointed out by Boyer, then an accentuation of the difference between the medium exchange reaction and the intermediate exchange might be obtained by studying ITP. ITP is hydrolyzed by myosin in the presence of magnesium at approximately 10 times the rate of ATP. Nevertheless, it was found to exchange about

the same amount of oxygen overall. Therefore, the medium exchange reaction should be drastically reduced for the same per cent hydrolysis since much less time is required. In Table 3 such evidence is presented. In this case the time required for 70 per cent hydrolysis was very short and during this time interval essentially no exchange occurred between $P_i O^{18}$ and H_2O^{16}. That is, the amount of exchange at the medium level was not detectable. On the other hand, in the experiment with H_2O^{18} where exchange at the intermediate level is detectable a figure of 0.76 atoms of O^{18} per total phosphate present was obtained. Using the same type of calculation used in the previous table, the exchange per phosphate group produced by hydrolysis of ITP is 1.9 atoms or essentially two out of the four atoms. These results therefore confirm the conclusion that exchange does, indeed, occur at the intermediate level.

To confirm this result further, another method of accentuating the intermediate exchange was performed. Dinitrophenol is known to activate the magnesium-catalyzed hydrolysis of ATP by myosin. Therefore, the ATP experiments were repeated but this time dinitrophenol was added so that the hydrolysis was essentially complete in 1.5 hours. The amount of exchange between $P_i O^{18}$ and H_2O^{16} was negligible (0.027) in the short interval (Table 4). During the same interval when H_2O^{18} and $P_i O^{16}$ were used, an exchange of 2.46 atoms per phosphate occurred. Again correcting for the small medium exchange it is possible to calculate that under these conditions 2.44 atoms of oxygen are exchanged per phosphate group at the intermediate stage in the reaction.

This system also allowed us to check our conclusions in regard to ATP which stood for longer intervals. Even though the hydrolysis of ATP was essentially complete in 1.5 hours we let the solution stand for 20 hours and the $P_i O^{18}$-H_2O^{16} medium exchange increased as to be expected for a time-dependent process.

Our conclusion from these studies therefore is that we have been able to confirm Dempsey and Boyer's discovery of a medium exchange between the P_i of the medium and the oxygen in the water catalyzed by myosin during ATP hydrolysis. However, we disagree in the finding that this exchange can account for all of the O^{18} exchange occurring during hydrolysis of ATP or ITP by myosin. In our routine experiments the amount of exchange occurring at the intermediate stage is appreciably higher than that occurring at the medium level and under special circumstances, for example, with a rapidly hydrolyzing nucleotide such as ITP or in the presence of an activated ATP hydrolysis, i.e. with dinitrophenol, essentially no medium exchange at all is observed in an interval when intermediate exchange occurs readily.

The reason for the discrepancy in these two laboratories is not yet understood. It has been our pleasure to have a most cooperative and friendly exchange with Dr. Boyer. From his data above we would certainly be led to the same conclusions which he has obtained. It would seem therefore that one of several possibilities exists. a) an inhibitor is present in his preparation. b) an activator is present in our preparation. c) some basic modification at the protein has occurred in one laboratory or the other to lead to these rather different results. We had found under other circumstances, for example, in the presence of

barium, that no O^{18} exchange occurs.(4) Thus the O^{18}-exchange reaction must depend on some general features of the active site and these features do not necessarily parallel nucleotide triphosphate hydrolysis. Thus it is not unreasonable that an inhibitor or some modification of the protein itself would change the conformation at the active site in such a way that the ATPase activity would be retained but the O^{18}-exchanging reaction would be decreased.

Having convinced ourselves that an O^{18} exchange reaction did indeed occur at an intermediate stage we have used this property to study some of the properties of the protein. Since these studies have been reported elsewhere(7) or are shortly to be reported(8), I will only summarize them very briefly. The most striking conclusions are that the properties of the O^{18} exchange reaction do not parallel the properties of the hydrolysis rate. The metal ion dependence of the two processes are different, the pH dependence of the two processes are different, the nucleotide specificity of the two processes are different, and the temperature dependence of the two processes are different.

On the other hand, there are some striking similarities between the O^{18} exchange specificity and the specificity for contraction. For example, the nucleotides which show the highest O^{18} exchange are the most effective nucleotides for contraction. The metal ions showing the highest O^{18} exchange are the most effective metal ions for contraction. It seems therefore that there are some properties of the active site which are essential for the O^{18} exchange reaction which are not essential

for the overall rate of hydrolysis. These properties have certain strong similarities to the requirements for contraction. One can conclude that some features of the active site which are essential for contraction can be probed by this O^{18} exchange reaction and can lead to a greater understanding of the relation of the hydrolysis to the mechanism of muscular contraction.

REFERENCES

(1) Koshland, D. E., Jr., Budenstein, Z. and Kowalsky, A., J. Biol. Chem. 211, 279 (1954).

(2) Gergely, J. and Maruyama, K., J. Biol. Chem. 235, 3174 (1960).

(3) Koshland, D. E., Jr. and Clarke, E., J. Biol. Chem. 205, 917 (1953).

(4) Levy, H. M. and Koshland, D. E., Jr., J. Biol. Chem. 234, 1102 (1959).

(5) Dempsey, M. E. and Boyer, P. D., J. Biol. Chem. 236, PC6 (1961).

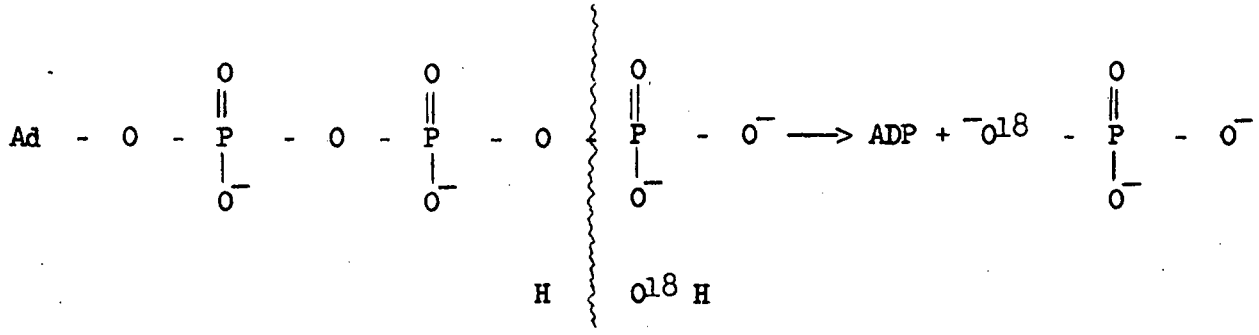
(6) Levy, H. M., Ryan, E. M., Springhorn, S. S. and Koshland, D. E., Jr., J. Biol. Chem. 237, PC1730 (1962).

(7) Levy, H. M., Sharon, N., Lindemann, E. and Koshland, D. E., Jr., J. Biol. Chem. 235, 2628 (1960).

(8) Yount, R. G. and Koshland, D. E., Jr., unpublished.

Table 1

Oxygen Exchange Catalyzed by Myosin and Actomyosin



System	O^{18} Atoms Per Phosphate Group	
	ADP	P_i
Myosin	None	3.4
Actomyosin	None	2.8

Conditions: 0.125 M Tris, pH 7.4, 0.10 M KCl, 0.01 M $MgCl_2$, 0.01 M ATP; 2-6 mg protein/ml.; 25° C.

Table 2

Oxygen Exchange During ITP Hydrolysis

Conditions: 0.125 M Tris, pH 7.4, 0.10 M KCl, 0.01 M $MgCl_2$, 0.005 M P_i, 0.005 M ITP, 2-5 mg protein/ml, 25° C.

Expt. No.	Initial Position of O^{18}	Per Cent Hydrolysis	Oxygen Atoms Exchanged/Phosphate Ion Designated		
			Initially Added P_i	Total P_i Present*	Calculated for Exchange at Intermediate Stage
1	P_i	27	0.10		
2	P_i	95	0.02		
3	Water	71		0.76	1.92
4	Water	82		0.76	1.78

* P_i added initially plus P_i produced by hydrolysis

Table 3

Oxygen Exchange During ATP Hydrolysis Activated Five-fold with 0.03 M DNP

Expt. No.	Initial Position of O^{18}	Per Cent Hydrolysis	Oxygen Atoms Exchanged/Phosphate Ion Designated		
			Initially Added P_i	Total P_i Present*	Calculated for Exchange at Intermediate Stage
1	P_i	84 (1.5 hrs.)	0.027		
2	P_i	105 (20 hrs.)	0.45		
3	Water	90 (1.5 hrs.)		2.46	2.44
4	Water	120 (20 hrs.)		2.90	2.23

* P_i added initially plus P_i produced by hydrolysis

Table 4

Oxygen Exchange During ATP Hydrolysis

Conditions: 0.125 M Tris; pH 7.4; 0.10 M KCl, 0.01 M $MgCl_2$, 0.005 M P_i initially, 0.005 M ATP initially, 2-5 mg protein/ml, 25° C.

Expt. No.	Initial Position of O^{18}	Per Cent Hydrolysis	Oxygen Atoms Exchanged/Phosphate Ion Designated		
			Initially Added P_i	Total P_i Present*	Calculated for Exchange at Intermediate Stage
1	P_i	27	0.28		
2	P_i	108	0.56		
3	P_i	130	0.50		
4	Water	127		1.44	2.00
5	Water	130		1.40	1.85

* P_i added initially plus P_i produced by hydrolysis