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The Role of Nucleoside Triphosphate Hydrolysis in Transducing Systems: p21ras and Muscle [and Discussion]

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The role of nucleoside triphosphate hydrolysis in transducing systems: p21ras and muscle

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SUMMARY

A variety of systems use nucleoside triphosphate hydrolysis to control or provide energy for biological processes, mediated through protein–protein interactions. The nature of this coupling may vary, but often there is a degree of similarity. In this paper, two systems are compared: actomyosin in muscle and p21ras in a signal transduction pathway as yet undefined. The mechanism of the nucleotide triphosphate hydrolysis and the consequent changes in the protein–nucleotide complex have been investigated, to understand how the coupling to biological function is achieved. The basal nucleoside triphosphatase mechanisms are compared and the roles of proteins that activate the hydrolysis, actin and GAP, are discussed. The cleavage process was probed by stereochemical techniques to determine the basic mechanism, of either a phosphorylated enzyme intermediate or direct displacement of nucleoside diphosphate by water. Phosphate–water oxygen exchange probes were used to investigate nucleoside triphosphate and inorganic phosphate release steps. A new method of probing the kinetics of inorganic phosphate release directly has been developed. In muscle, this process seems likely to be related directly to force generation. In the GAP-ras system, measurement of phosphate release is allowing the mechanism of the GAP-p21ras interaction to be probed.

1. INTRODUCTION

A common feature of most, if not all, transducing GTPase and ATPase systems is that the energy of the nucleoside triphosphate hydrolysis is used to drive a cycle of protein–protein, or protein–ligand interactions. In terms of biological function, there is a cycle of activation and deactivation. In this paper, I examine some common features and some differences between a typical system that uses ATP, actomyosin of muscle, and one that uses GTP, p21ras. This will concentrate on the molecular mechanism of these systems, and the way in which different nucleotide states interconvert, so producing the cycle of activity.

Actomyosin is relatively well understood, although the details of how the ATP hydrolysis is coupled to mechanical force production remain elusive. The mechanism of the myosin and actomyosin ATPase have been investigated widely (for reviews see Trentham *et al.* (1976); Taylor (1979); Hibberd & Trentham (1986)). A simplified mechanism is shown in figure 1 and this has the main features shared by actomyosin in solution and in muscle fibres. ATP binding induces dissociation of actin from myosin. Hydrolysis can occur either with the proteins associated or dissociated with similar rate constants, but following hydrolysis the proteins interact strongly again. In contracting muscle the cycle must include a period of dissociation in order for myosin filaments to move relative to actin. Release of inorganic phosphate seems intimately related to force generation in muscle fibres (Hibberd *et al.* 1985). The scheme in figure 1 does not include all protein conformation changes that are likely to be important in the transduction. The very nature of this transduction, to produce force in a muscle, makes the molecular mechanism difficult to study. Both myosin and actin are organized in filaments, with associated control proteins. The development of 'skinned' fibre preparations has made the study of the details of the transduction possible, by comparing mechanical and biochemical responses.

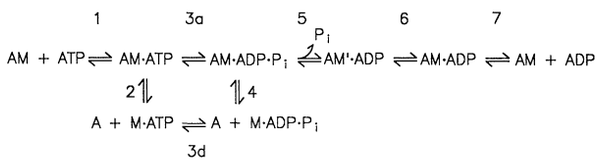

Figure 1. Mechanism of actomyosin ATPase reaction. A represents actin, M myosin. Steps are numbered such that step i has forward and reverse rate constants k_{+i} and k_{-i}. Two AM.ADP states are shown as proposed by Sleep and Hutton (1980).

The ras system is well studied in many ways, partly because several single point mutants of p21ras are oncogenic and partly because this protein is an archetype for a rapidly growing superfamily of low molecular mass (15–25 kDa), guanine nucleotide binding proteins (Bourne *et al.* 1990, 1991). These proteins share considerable sequence homology, bind

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GDP or GTP tightly and have low GTPase activity. Members of this superfamily are implicated in the control of cellular functions, such as signalling and trafficking, and are presumed to work via a cycle of activation to the GTP-bound state and deactivation to the GDP-bound state. In the case of ras, these interconversions are greatly stimulated by 'GTPase activating proteins' (GAPs) (Trahey & McCormick 1987; Ballester *et al.* 1990; Martin *et al.* 1990; Xu *et al.* 1990) and nucleotide exchange stimulating proteins (de Gunzburg *et al.* 1989; Downward *et al.* 1990; Crechet *et al.* 1990; Huang *et al.* 1990; Mizunu *et al.* 1991). GAP may be part of an effector system or a down-regulator of p21ras (Hall 1990). However, a clear cellular function for this system has not been elucidated.

The mechanism of the GTP hydrolysis cycle is much less well understood than that for actomyosin. In the absence of activating proteins the GTPase activity is very low ($\approx 10^{-4}\,s^{-1}$ at 30°C) and most rate constants in the basal hydrolysis scheme (steps 1–4 in figure 2) have been measured (Neal *et al.* 1988). Two processes, hydrolysis and GDP release, have similar rate constants to the GTPase activity, and so unlike the situation with myosin, one activating protein increasing the rates of one elementary process will not increase the overall GTPase activity. Figure 2 shows a possible scheme including GAP, but the nucleotide exchange stimulating protein is not included, as little is known about the activated exchange or about the control of these proteins. Although GAP binds much tighter to the GTP-bound state of p21ras than the GDP-bound state, the sequence of events and most likely pathway is not known. So this scheme shows the possible involvement of GAP.p21 complexes with all nucleotide states.

The three p21ras proteins (H, N, K) have different post-translational modifications by addition of lipophilic groups at the C-terminal sequence (Gibbs 1991). These modifications are essential for membrane association and apparently also have functional importance. For example a nucleotide exchange stimulating protein has been isolated that acts only on modified protein (Mizunu *et al.* 1991). However, currently most work on the biochemistry of these systems uses proteins expressed, in *E. coli* that do not have the post-translational modifications.

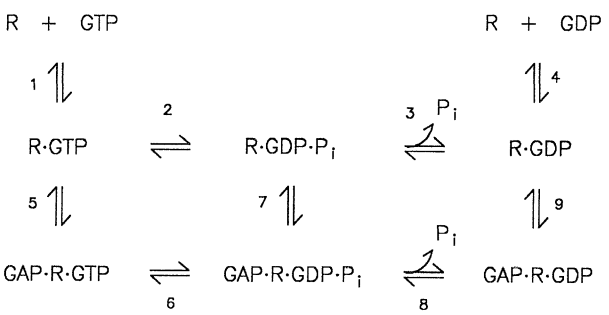

Figure 2. Mechanism of p21ras GTPase, showing the main possible interactions with GAP. R represents p21ras and elementary steps have rate constants as described for figure 1.

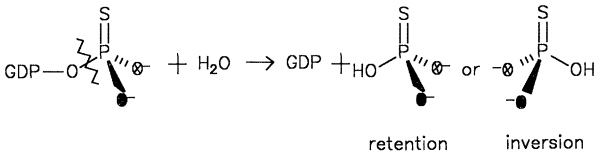

Figure 3. The possible stereochemical courses of the enzyme-catalysed hydrolysis of (γ-S) (γ-^{17}O,^{18}O)GTPγS. The products are the R and S isomers of inorganic (^{17}O,^{18}O)phosphorothioate. ^{17}O is represented by ⊠, ^{18}O by filled circles. The position of bond breaking (γ-P to bridging O) is shown by the wavy line.

2. CHEMICAL MECHANISM OF THE HYDROLYSIS

An important part of understanding the hydrolytic cycle is to determine the chemical mechanism of the cleavage step. The most likely alternatives are a reaction via a phosphoenzyme mechanism or direct displacement of nucleoside diphosphate by a water oxygen. This has been determined for both systems, by distinguishing the stereochemical course as shown in figure 3 (Webb & Trentham 1980*b*, 1982; Feuerstein *et al.* 1989). Each enzyme-catalysed phosphotransfer step (transfer of PO_3) inverts the configuration at phosphorus (Knowles 1980). So a single step mechanism shows overall inversion, a phosphorylated intermediate mechanism has two inversions, and so shows overall retention. By using a combination of oxygen isotopes and sulphur to label the terminal oxygens stereospecifically, the configuration of the inorganic phosphorothioate product can be determined by a nuclear magnetic resonance (NMR) technique, and hence the stereochemical course is defined (Webb & Trentham 1980*a*). This has been done for a wide range of ATPases and GTPases, and in almost all cases including myosin, actomyosin and p21ras, the result shows direct in-line displacement of nucleoside diphosphate by water (Webb 1982). So far the calcium pump ATPases is the only triphosphatase to show retention of configuration at phosphorus (Webb & Trentham 1981*b*) and this has a phosphoenzyme intermediate. Thus a single elementary step in figures 1 and 2 is a true representation of the cleavage process for actomyosin and for p21ras, although the experiment has not yet been done for the GAP-activated reaction.

3. PHOSPHATE–WATER OXYGEN EXCHANGE

It was mentioned in the Introduction that P_i release is intimately related to force generation in muscle. Is this type of relationship general for transducing systems and what is the importance of P_i release? One way to probe P_i release and the cleavage step is using phosphate–water oxygen exchange, a technique that has proved useful to obtain kinetic information for actomyosin in solution and in muscle fibres (reviewed by Sleep & Smith (1981); Webb & Trentham (1983)). This also provides an illustration of the different kinetics of the actomyosin and ras systems.

The mechanism of this exchange is shown in figure

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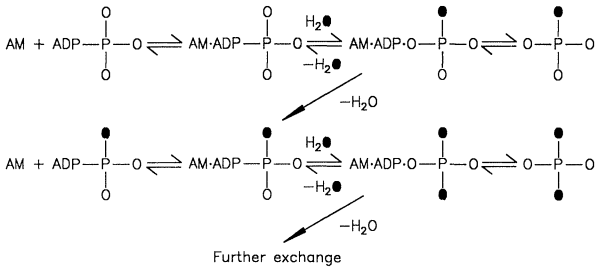

Figure 4. Mechanism of intermediate phosphate-water oxygen exchange during ATP hydrolysis by actomyosin. For simplicity this shows the mechanism only for when actin and myosin remain associated. ^{18}O is represented by filled circles.

4 for actomyosin. ATP binding is followed by cleavage, during which an ^{18}O is incorporated from water into P_i. This $(^{18}O)P_i$ can be released, or ATP can be resynthesized in the catalytic site. The relative importance of these pathways depends on the size of the two rate constants. On resynthesis of ATP one oxygen is lost to water and this may be ^{16}O, because the P_i can rotate in the catalytic site. A repeat hydrolysis causes a second ^{18}O to be incorporated. Eventually all four oxygens in P_i may be ^{18}O and the relative proportions of the different labelled species depends on k_{+4}/k_{-3}. The normal method of measurement of the amount of ^{18}O in the P_i is by mass spectrometry on a volatile derivative.

This technique has been widely used with actomyosin and with muscle fibres. Figure 5 shows a simple experiment with myosin subfragment 1 in the absence and presence of actin. In the absence of actin almost all P_i has four solvent oxygens. k_{+4}/k_{-3} is large and

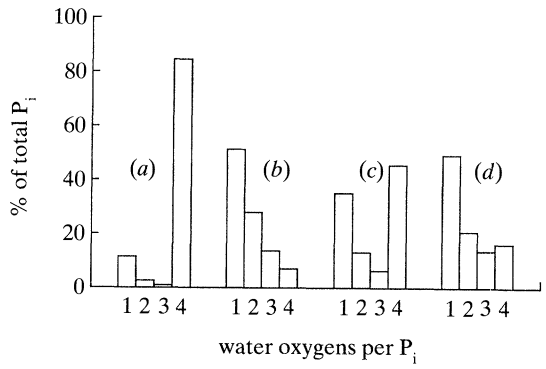

Figure 5. Distribution of oxygens derived from water following ATP hydrolysis by: (*a*) myosin subfragment 1; (*b*) actomyosin subfragment 1; (*c*) a demembranated rabbit psoas fibre in the absence of Ca^{2+}; (*d*) a fibre in the presence of Ca^{2+}. The conditions were for (*a*) and (*b*): 10 mM *N*-tris(hydroxymethyl)methyl-1,2-aminoethane sulphonic acid (TES) pH 7.0, 5 mM ATP, 6 mM $MgCl_2$, 10 mM KCl, 0.1 mM dithiothreitol, 22°C. In (*b*) there was 36.4 μM F-actin and the myosin subfragment 1 concentration was such that ≈50% hydrolysis occurred in 10 min. For (*c*): 100 mM TES, pH 7.0, 8 mM MgATP, 30 mM EGTA, also containing 1,6-diaminohexan-N,N,N′,N′-tetra-acetic acid to give an ionic strength of 200 mM. The solution for (*d*) also contained Ca^{2+} to give free $[Ca^{2+}]$ of 32 μM. A single fibre was incubated in this solution in (^{18}O)water as described by Bowater *et al.* (1989).

ATP is re-synthesized many times before P_i is released. As actin increases, there is almost no effect on k_{-3} but k_{+4} increases, so at high actin there is much less exchange (Shukla & Levy 1977; Sleep & Boyer 1978). This is reflected at least qualitatively in skinned muscle fibres (figure 5) (Hibberd *et al.* 1985; Bowater *et al.* 1989). When the fibre is relaxed in the absence of Ca^{2+}, there is little interaction between myosin and actin filaments, and the exchange is qualitatively similar to that for subfragment 1 although more complex with a component showing little exchange. In the presence of Ca^{2+}, the fibre produces tension. The pattern of exchange is qualitatively similar to actomyosin and shows k_{+4} to have increased. A detailed study of the exchange in activated fibres suggests a model in which k_{-1} and k_{+4} (see figure 1) are dependent on the strain of the myosin molecules attached to actin (Bowater *et al.* 1989) in contrast to solution measurements where proteins are unstrained.

This type of oxygen experiment has been done with p21ras, starting with the R.GTP complex. The product shows almost no exchange, indicating P_i release (k_{+3} in figure 4) is much faster than GTP resynthesis (k_{-2}). There is a small amount of exchange that fits with a model in which P_i rebinds to R.GDP, synthesizes R.GTP, which then breaks down to R.GDP and P_i that has gained a solvent oxygen.

If the experiment is done starting with R.GDP and incubating with $(^{18}O_4)P_i$, a slow loss of ^{18}O is observed with at most one ^{18}O lost each time P_i binds. Thus p21ras behaves opposite from myosin in this respect: $(^{18}O_4)P_i$ binds to myosin subfragment 1 and loses all four oxygen-18 atoms before it is released from the catalytic site (Sleep *et al.* 1978; Webb *et al.* 1978). If the same experiment is done with p21ras in the presence of GAP, almost no change is observed from the measurement in the absence of GAP for conditions where the hydrolysis is accelerated some 200-fold. Assuming P_i release is unaffected by GAP, no effect of GAP is observed on GTP resynthesis. This requires further investigation, particularly at high GAP activation, but the results do provide support for a model in which there is a conformation change prior to the hydrolysis step (figure 6) (Neal *et al.* 1990; Eccleston *et al.* 1992), with the main effect of GAP being to accelerate the conformation change.

4. PHOSPHATE RELEASE KINETICS

To investigate the role of the cleavage step and P_i release further, it is necessary to have a direct way of measuring them. My laboratory has been developing methods for following the kinetics of P_i release that could be used for actomyosin ATPase in solution, GAP-activated p21ras, and skinned fibre preparations.

$$R{\cdot}GTP \overset{2a}{\rightleftharpoons} R^{*}{\cdot}GTP \overset{2b}{\rightleftharpoons} R{\cdot}GDP{\cdot}P_i$$

Figure 6. Two step hydrolysis mechanism for p21ras. A slow conformation change to the GTP complex is followed by a more rapid cleavage step.

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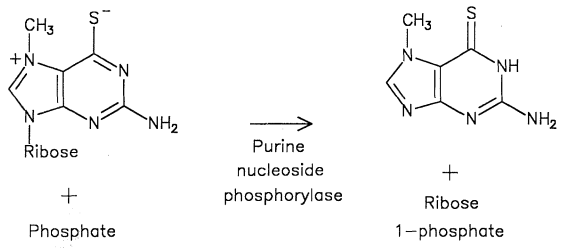

Figure 7. Reaction of 2-amino-6-mercapto-7-methyl purine ribonucleoside with P_i catalysed by purine nucleoside phosphorylase. The nucleoside is a methyl-thio-analogue of guanosine (MESG).

One method is illustrated in figure 7, using a guanosine analogue, 2-amino-6-mercapto-7-methyl-purine ribonucleoside (MESG) (Brown & Milne 1975), which is a substrate for purine nucleoside phosphorylase (Webb 1992). This phosphorolysis produces an absorbance increase at 360 nm, in the range pH 6.5–8.5, allowing P_i in the micromolar range to be measured. The K_m for P_i is 26 μM and the k_{cat} is 40 s^{-1} (25°C, pH 7.5). The methodology is illustrated by the measurement of P_i release during ATP hydrolysis by myosin subfragment 1 (figure 8). P_i release is measured as an increase in absorbance during the concomitant reaction of MESG with the P_i. This occurs at the rate constant for the ATPase activity: P_i release is rate limiting (Trentham *et al.* 1972). By coupling this reaction to the GTP hydrolysis on p21ras, this system has been used to probe GAP activation (M. R. Webb & J. L. Hunter unpublished result). The method is providing a simple way of measuring steady state interactions of GAP with p21ras, both wild-type and mutants. We are currently using it in combination with stopped-flow apparatus to test the role of P_i release in these two systems. For example, does P_i release become rate limiting at high GAP activation? How does P_i release in muscle fibres correlate with force generation?

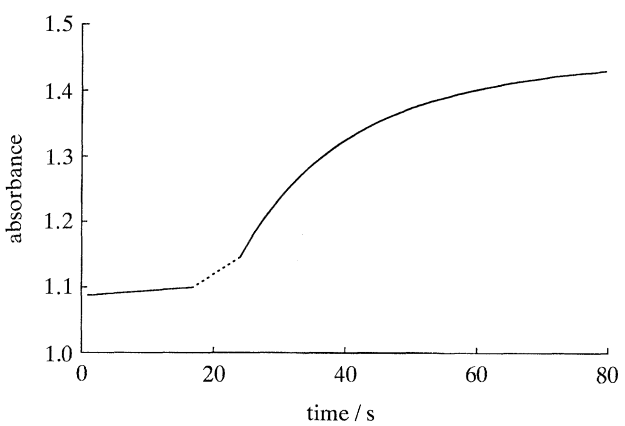

Figure 8. Single turnover release of P_i from myosin subfragment 1 during ATP hydrolysis, as measured by the MESG/phosphorylase system. The solution at 30°C, monitored at 360 nm in a 1 cm cuvette, contained 20 mM Tris-HCl buffer PH 7.5, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.2 mM MESG, 12.5 unit. ml^{-1} purine nucleoside phosphorylase, 34 μM subfragment 1. The reaction was initiated by addition of 31 μM ATP. The data fit a single exponential with a rate constant 0.06 s^{-1}. The absorbance increase before addition of ATP (which occurs during the dashed line) is due to a slow non-enzymic reaction.

5. DISCUSSION

It is possible that for both p21ras and myosin the activating proteins (GAP and actin) stimulate hydrolysis, not due to an effect on the chemical cleavage step, but through acceleration of a neighbouring process: P_i release for actin, a prior conformation change for GAP. This may have interesting consequences for the enzymology of such transducing systems. The array of amino acids in the catalytic site may be equally able to produce catalysis with or without the activator. The role of the activation (considered in terms of the triphosphatase) is to change a non-chemical step (or steps), eventually allowing the unchanged chemical step to become rate limiting. In both cases, the degree of activation is very dependent on ionic strength, with the binding between proteins decreasing as ionic strength increases.

It is difficult to compare the kinetics of the hydrolysis step of these two systems because equivalent measurements are not possible with the two systems. The maximum activated rate constant for hydrolysis is ≈20 s^{-1} at 37°C for the GAP-ras system (John *et al.* 1990) and so this represents the lower limit for the cleavage rate constant. For myosin k_{+3} ≈80 s^{-1} at 20°C (Webb & Trentham 1981*a*) and this rate constant is probably not much affected by activation by actin (Rosenfeld & Taylor 1984; Ferenczi 1986). In terms of either thermodynamics or activation from free solution hydrolysis of ATP or GTP, these are very similar catalytic activities.

Myosin and p21ras share with a range of ATPases and GTPases considerable sequence homology on the catalytic site, particularly in the phosphate binding region (Saraste *et al.* 1990). Electron paramagnetic resonance (EPR) studies indicate that Mg^{2+} may bind to the β- and γ-oxygens of the triphosphate moiety (Webb *et al.* 1982). The crystal structure of p21ras show βγ binding here also (Wittinghofer & Pai 1991). For both systems, the diphosphate is likely formed as the β complex. This information taken with the shared in-line mechanism, and position of bond breaking (figure 2) may indicate that the chemical mechanism is very similar for the two systems, and may be mediated by a similar array of amino acids in the catalytic site.

Nucleotide exchange is controlled by nucleoside diphosphate release at physiological nucleoside triphosphate concentrations. As outlined above, an exchange factor accelerates this process for p21ras.GDP. It is currently not clear whether GAP and the exchange factor are part of the signal pathway, or are control proteins. There is no separate exchange factor for myosin and actin remains bound relatively tightly throughout the cycle except at the weak binding states at the cleavage. The presence of

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actin accelerates ADP release more than 100-fold (from $\approx 1\ s^{-1}$ in the absence of actin), so that this process is unlikely to contribute to rate limitation (Trentham *et al.* 1976; Geeves 1989). In effect, actin acts as a 'nucleotide stimulating exchange protein'.

The change in nucleotide states of either system modulates the interaction with activators, actin and GAP. For actomyosin, it seems that P_i release is associated with an increase in affinity of actin and myosin, although the sequence of events remains uncertain and this is also associated with force generation. For p21ras, it is not known whether the R.GDP.P_i and subsequent P_i release play a role in decreasing the affinity of GAP, or whether this is achieved before this intermediate, associated with the cleavage itself or a prior conformation change. Direct measurements of P_i release should clarify these questions for both systems.

I thank my co-workers who have contributed to the work described here and whose published work is mentioned in the References.

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Discussion

D. R. Trentham (*National Institute for Medical Research, Mill Hill, London, U.K.*). Is there any evidence now, either from Heidelberg or from other groups about the equilibrium constant of phophate binding to the ras GDP complex?

M. R. Webb (*National Institute for Medical Research, Mill Hill, London, U.K.*). We get a K_m value from measuring P_i–water oxygen exchange of a few millimolar, but that is not an exact number.

A. G. Weeds (*MRC Laboratory of Molecular Biology, Cambridge, U.K.*). Can Dr Webb fill us in on the evidence that the ras GTPase has these several hypothesized states before cleavage? Are there any signals like there are with the myosin ATPase that give this evidence?

M. R. Webb. Dr John Eccleston's paper will give the evidence for that, which is based on fluorescence measurements.

A. G. Weeds. I thought there was one step in Dr Webb's mechanism which refers back to Dr Goody's talk in which his GDP off rate was 10^{-5} s^{-1} which is slower than Dr Webb's 10^{-4} s^{-1}.

R. S. Goody (*Max-Planck-Institut für medizinische Forschung, Heidelberg, F.R.G.*). There is another isomerization which we found, also using fluorescence techniques which occurs on GTP binding at a step before the slower isomerization which Dr Webb has mentioned. It is the reverse of that which is 10^{-5} s^{-1}. In the forward direction the rate constant is about 10–20 s^{-1}.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES