Stabilization of the ADP/Metaphosphate Intermediate during ATP Hydrolysis in Pre-power Stroke Myosin

**QUANTITATIVE ANATOMY OF AN ENZYME**

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**Background:** It is unknown how enzymes actually achieve the catalytic pathways proposed for ATP hydrolysis.

**Results:** A quantum mechanical analysis of myosin ATPase quantifies the relevant interactions that stabilize a metaphosphate intermediate.

**Conclusion:** The protein is designed to stabilize this metaphosphate, key to the enzymatic mechanism.

**Significance:** This yields a chemically consistent model for the catalytic strategy of nucleotide-hydrolyzing enzymes in general.

It has been proposed recently that ATP hydrolysis in ATPase enzymes proceeds via an initial intermediate in which the dissociated γ-phosphate of ATP is bound in the protein as a metaphosphate (P<sub>γ</sub>O<sub>3</sub>). A combined quantum/classical analysis of this dissociated nucleotide state inside myosin provides a quantitative understanding of how the enzyme stabilizes this unusual metaphosphate. Indeed, in vacuum, the energy of the ADP<sup>3-</sup>-PO<sub>3</sub>-Mg<sup>2+</sup> complex is much higher than that of the undissociated ATP<sup>3</sup>-<sup>−</sup>. The protein brings it to a surprisingly low value. Energy decomposition reveals how much each interaction in the protein stabilizes the metaphosphate state; backbone peptides of the P-loop contribute 50% of the stabilization energy, and the side chain of Lys-185<sup>+</sup> contributes 25%. This can be explained by the fact that these groups make strong favorable interactions with the α- and β-phosphates, thus favoring the charge distribution of the metaphosphate state over that of the ATP state. Further stabilization (16%) is achieved by a hydrogen bond between the backbone C=O of Ser-237 (on loop Switch-1) and a water molecule perfectly positioned to attack the P<sub>γ</sub>O<sub>3</sub> in the subsequent hydrolysis step. The planar and singly negative P<sub>γ</sub>O<sub>3</sub> is a much better target for the subsequent nucleophilic attack by a negatively charged OH<sup>−</sup> than the tetrahedral and doubly negative P<sub>γ</sub>O<sub>3</sub><sup>−</sup> group of ATP. Therefore, we argue that the present mechanism of metaphosphate stabilization is common to the large family of nucleotide-hydrolyzing enzymes. Methodologically, this work presents a computational approach that allows us to obtain a truly quantitative conception of enzymatic strategy.

Adenosine triphosphate (ATP) is the universal energy currency of biology (1, 2) in the form of its hydrolytic decomposition into adenosine diphosphate (ADP) and inorganic phosphate (see Fig. 1). ATP is very stable in solution, as indicated by its low rate of spontaneous hydrolysis (with rate constants of 3.2 × 10<sup>−5</sup> s<sup>−1</sup> and 17.5 × 10<sup>−5</sup> s<sup>−1</sup> at pH 8.4 and pH 4–5, respectively) (3, 4). These rates correspond to an activation free energy barrier in the 23–24 kcal mol<sup>−1</sup> range (5). The energy barrier for the associative and dissociative methyl triphosphate hydrolysis in vacuum was calculated to be 40.0 and 42.0 kcal mol<sup>−1</sup>, respectively. Nevertheless, ATP hydrolysis is the most frequent chemical reaction occurring in the human body, (7) because it is catalyzed by ATPases (8, 9) to provide the energy required for most cellular processes (10, 11, 12). ATPases are enzymes that lower the energy barrier of ATP hydrolysis to as low as 10–15 kcal mol<sup>−1</sup>, thus allowing for a fast turnover rate (on the millisecond time scale). Much effort has gone into studying the catalytic mechanism of ATPases, (3, 13–18), but the catalytic strategy of these enzymes remains largely controversial. A Car-Parrinello molecular dynamics simulation (19) has shown that, in water, the dissociative mechanism (P–O bond cleavage precedes the attack of the hydrolyzed water; Fig. 1B) is slightly preferred over the associative mechanism (nucleophilic attack and breaking of the P<sub>γ</sub>–O<sub>βγ</sub> bond are concerted; Fig. 1A). Recent quantum mechanical investigations of F<sub>1</sub>-ATPase (15) and myosin-ATPase (16, 17) have proposed a dissociative mechanism (Fig. 1B), in which the initial event in the enzyme is the cleavage of the bond between the P<sub>γ</sub> and the oxygen O<sub>βγ</sub> of the triphosphate moiety prior to attack of the P<sub>γ</sub> phosphorus by a water molecule. This results in the formation of a planar PO<sub>3</sub><sup>−</sup> metaphosphate, which is bound in the protein between the ADP<sup>3−</sup>, the Mg<sup>2+</sup> ion, and the attacking water. In both studies, the energy of the protein in this metaphosphate state (henceforth called P<sub>m</sub><sup>3</sup>) is found to be quite low, only 8.1 kcal mol<sup>−1</sup> (myosin) (16, 17) to 14.5 kcal mol<sup>−1</sup> (F<sub>1</sub>-ATPase) (15) above the ATP reactant state. This is surprising, given that the hydrated ADP<sup>3−</sup>-PO<sub>3</sub><sup>−</sup>-Mg<sup>2+</sup> complex is very unstable in

<sup>2</sup>Using B3LYP density functional with a 6-31+G** basis set, we computed the hydrolysis barrier of methyl triphosphate<sup>4−</sup> complexed with Mg<sup>2+</sup> in the presence of four Mg<sup>2+</sup>-coordinated water molecules and one attacking water molecule.

<sup>3</sup>The abbreviations used are: P<sub>m</sub> metaphosphate state of protein with PO<sub>4</sub><sup>−</sup> and ADP bound; W<sub>α</sub> and W<sub>β</sub> attacking and helping water molecule, respectively; QM, quantum mechanical; MM, molecular mechanical; H-bond, hydrogen bond.
the absence of the proteins. Neither study explains how the enzymes manage to bring the energy of the P_m state to such low levels. Moreover, the two studies disagree about whether the P_m state is a local energy minimum (as found for F_1-ATPase) (15) or rather an unstable transition state structure (as claimed for myosin) (16, 17). Notwithstanding, we propose that the formation of the P_m state is central to the whole catalytic strategy of ATPases and P–O bond-hydrolyzing enzymes in general. Indeed, the PO_3^2− is planar and has only a single negative charge, which makes it a much better target for attack by an OH^− hydroxyl group than the tetrahedral and doubly charged P_2O_5^2− of ATP (Fig. 1, compare A and B). Therefore, the stabilization of the P_m state emerges as a key element of the enzymatic mechanism. The questions addressed here are the following. 1) What is the energy of the P_m state, and is it a stable or rather a metastable intermediate? 2) How can the protein achieve this stabilization? 3) What are the protein residues responsible for P_m stabilization, and how much do they respectively contribute? We answer these questions by analyzing the P_m state in the myosin ATPase, using combined quantum/classical (QM/MM) calculations with density functional theory at a highly accurate level.

Myosin II is a molecular motor responsible for muscle contraction (20, 21). It uses the energy derived from ATP hydrolysis to drive the cyclic interactions between myosin and the actin filament that produce motion (Lynn-Taylor cycle) (20). During this cycle, myosin undergoes several conformational changes, which involve the successive closing of two loops (called Switch-1 and Switch-2; see Fig. 2A) around the ATP binding site, leading to a catalytically active ATPase (22–24). In this conformation, the rebinding of myosin to the actin filament and the concurrent power stroke can only occur after ATP has been hydrolyzed. We refer hereafter to this pre-power stroke conformation with ATP bound as the “reactant” state of the hydrolysis reaction. In this state, and looking at myosin-2 of Dicystostelium discoideum, the triphosphate moiety of ATP is tightly held by 15 hydrogen bonds with the surrounding protein (Fig. 2A). The attacking (W_a)^3 and a helping (W_h) water are positioned by a dense hydrogen bonding network between the ATP and loops Switch-1 and Switch-2. Starting from this reactant state, the experimental rate of catalyzed hydrolysis is $k_{\text{cat}} = 10^3$ s⁻¹, which is 10⁶ times faster than in solution and corresponds to a free energy barrier of 14.4 kcal mol⁻¹ (25).

We find that the structure of the P_m state is very similar to the crystallographic reactant state, the only major difference involving a 1.1-Å displacement of the γ-phosphorus atom (see Fig. 2B) as the substrate dissociates into ADP³⁻ and P_2O_5$. The P_2O_5 is close to the W_a attacking water (PγO_W₆A distance is 1.95 Å), as had been observed in the P_m state of F_1-ATPase (15), thus forming a hydrate complex. Our calculations show that in the absence of the protein, the energy of the substrate is 44.3 kcal mol⁻¹ higher in the P_m than in the reactant state. In the presence of myosin, this energy difference is reduced to 2.3 kcal mol⁻¹. To understand this strong stabilizing effect from the protein, the electrostatic interactions of the protein with the substrate in the reactant state were compared with those in the P_m state. Moreover, those interactions were decomposed into contributions from each protein moiety surrounding the substrate. With this approach, it was possible to pinpoint which groups are responsible for the stabilization and to precisely quantify their contribution. We identified eight peptide groups and three side chains that contribute most of the stabilizing interactions (Table 1). For example, the single most prominent peptide interaction is the H-bond between the backbone C=O of Ser-237 (on loop Switch-1) and the attacking water, which lowers the energy of the P_m state (relative to the reactant state) by 12 kcal mol⁻¹, thus accounting by itself for 16% of the overall stabilization effect. Interactions with the backbone of the P-loop (residues 182–187) contribute ~50% of the stabilization by forming hydrogen bonds with the oxygens of the α- and β-phosphates. Among the side chains, Lys-185 induces a major stabilizing effect (25% of the overall effect) by improving its negative charge from the γ-phosphorus atom (see Fig. 1A) to the α- and β-phosphates in the P_m state. More stabilizing interactions are listed in Table 1. Together, these form a pattern from which the catalytic strategy of the protein clearly emerges. Most of these interactions exploit the shift of one negative charge from the γ-phosphate in the reactant substrate (see Fig. 1A) to the α- and β-phosphates in the P_m substrate (Fig. 1B). Meanwhile, the Ser-237 carbonyl polarizes the W_a, thus promoting a stabilization of the P_2O_5-H₂O₆W₆A hydrate complex. The resulting metaphosphate species is then a much improved target for the attack by the nucleophilic OH⁻ group.

### TABLE 1

| Protein residue | Protein moiety | Substrate moiety | ΔΔE | Percentage of total |
|-----------------|----------------|-----------------|------|---------------------|
| Gly-182         | Backbone NH    | O_p             | −8.2 | 11 (17) *          |
| Ala-183, Gly-184, Lys-185, Thr-186, Glu-187 | Backbone NH | O_p, O_p, O_p, O_p | −26.6 | 35  |
| Ser-237         | C=O            | Hydrogen of W₆a | −12.0 | 16  |
| Gly-157         | C=O            | Hydrogen of W₆n | −3.9  | 5   |
| Lys-185         | NH₂⁺           | O_p, O_p, O_p, O_p | −18.9 | 25  |
| Asn-233         | Side chain NH₂ | W₆⁺–P–O₃⁻ hydrate | −3.4  | 4   |
| Ser-236         | −OH            | NA*             | +24.1 | NA* |
| Arg-238         | Guanidinium     | NA*             | −13.9 | NA* |
| Glu-459         | −COO           | NA*             |      |      |

*ΔΔE is the free energy barrier of transition from reactant state to metaphosphate state.

*Percentage contribution from the Gly-182 backbone NH when its quantum effects on stabilization (~5.08 kcal mol⁻¹; see Table 2) are added to the ΔΔE.

*NA, not applicable.
resulting from the subsequent hydrolysis of water \( W_m \). Exactly equivalent interactions are made in the structures of other nucleotide-hydrolyzing enzyme, such as Ras-RasGAP or the F$_1$-ATPase (see “Discussion”), indicating that the metaphosphate-stabilizing strategy demonstrated here for myosin is used by other phosphate-hydrolyzing enzymes.

**MATERIALS AND METHODS**

**Computational Setup**—The protein structure and classical energy function used here are mostly identical to those described in our previous QM/MM study of ATPase in myosin (5, 25). The structure of the protein was taken from a crystal of myosin complexed to the ATP analog ADP-BeF$_3$. The positions of the \( W_m \) and \( W_h \) molecules were taken from the crystal structure for the complex with ADP$_3$(PO$_4$)$_3$ (Protein Data Bank entry 1VOM) (26) as well as waters 1581 and 1178 of 1VOM. The side chain orientations of Glu-187, Asn-219, and Lys-241 were also taken from the 1VOM structure. The protein was divided into four concentric regions: 1) a central region composed mostly of the substrate, in which the atoms are QM-treated (all other atoms are treated classically); 2) a surrounding region consisting of all atoms within 14 Å of the γ-phosphate (these atoms are free to move); 3) a region of 8 Å around the previous region, in which a harmonic force (constant of 0.05 kcal mol$^{-1}$ Å$^{-2}$) is applied to each atom to keep it near its position in the crystal; and 4) a region consisting of all remaining atoms, which remain fixed in their positions in the crystal. The solvent screening of electrostatic interactions with the classical atoms was accounted for by using non-uniform charge scaling, an implicit solvent method described previously (27). In non-uniform charge scaling, the charges of the classically treated atoms are rescaled in such a way that their mutual electrostatic interactions reproduce the corresponding interactions obtained from a solution of the Poisson-Boltzmann equation.

The main methodological difference relative to our previous study was the choice of the quantum method. Here we use the hybrid density functional B3LYP (28, 29) with the 6–311+G** basis set. The classical atoms were subjected to the CHARMM force field, using parameters described previously (30, 31). QM/MM boundaries were treated using the link atom approach, as described previously (32, 33). Another difference is the choice of the QM region, which was extended. The QM region consisted of 84 atoms. These include the triphosphate moiety of ATP and the functional side chain groups of Ser-181, Thr-186, Ser-236, Ser-237, Arg-238, and Gly-459 residues. For residues Thr-186, Ser-236, and Ser-237, the link atom was placed between the \( C_q \) and \( C_p \) atoms. In the case of Gly-459, the link atom was placed between \( C_q \) and \( C_p \). For Arg-238, the link atom was placed between \( C_q \) and \( C_p \). The peptide group [COC$_1$]-[Ser-181][NH$_2$]$_{Gly-182}$ was also included in the QM region. When a peptide is taken into the QM region, the point charges on the two neighboring peptide groups were modified as shown in Fig. 3. This avoids having a non-zero partial atomic charge on the two classical atoms making a bond to the QM peptide (i.e. Cedi$-1$) and C(i + 1) in Fig. 3) while preserving the dipole moments of these two peptide groups.

**Polarization Factors**—Peptide groups are known to get easily polarized (i.e. their dipole moment significantly increases when they are in contact with a moiety carrying a net charge (34–37). Thus, some of the peptide groups making hydrogen bonds with the triphosphate moiety (which carries a net charge of $-4e$) are expected to get highly polarized. In some cases, this can affect the energy difference \( \Delta E \) between the reactant and the \( P_m \) state. For example, taking peptide 182/183 quantitatively instead of classically further stabilizes the \( P_m \) state by \( \Delta E(182/183) = -1.24 \) kcal mol$^{-1}$. The second column in Table 2 shows this energy change, \( \Delta E(i) \), for each peptide group contacting the triphosphate. Ideally, all of the peptide groups around the triphosphate would be treated quantum mechanically, because this allows for the necessary polarization of their electron density. However, this would add so many atoms into the QM region that it is not computationally feasible. Therefore, the polarization was accounted for here by individually scaling up the classical atomic charges of each peptide. The charges of peptide group $i$ are multiplied by a polarization factor \( P_F(i) \) (given in the rightmost column).
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iteratively until a self-consistent set of PF_{i(o)} values was obtained. The resulting set of the self-consistent polarization factors, PF_{w(i)}, is listed in column 4 of Table 2. When |ΔE_{w(i)}| is less than 0.5 kcal mol^{-1}, then the standard charges of peptide i were left unchanged (i.e., PF_{w(i)} = 1 (e.g., for peptide 183/184). These PF_{w(i)} values were used to multiply the atomic point charges of their respective peptides during the geometry optimization of the reactant and the Pm structures. The energy was minimized to a gradient of <0.01 kcal mol^{-1} Å^{-1}, using the Turbomole version 5.9 program package (38) interfaced to CHARmm version c28b (39).

Electrostatic Interactions with the Substrate—In order to quantify the amount of stabilization of the Pm state due to electrostatic interaction of individual protein moieties (Figs. 4 and 6), the QM region was reduced so as to contain only the substrate (defined under "Results" and shown in Fig. 2B), totaling 45 QM atoms. In this case, peptide group 181/182 was treated classically, and its atomic charges were upscaled by a factor of 1.7 to partially account for its polarization. The geometries of the reactant and the Pm structures were then energy-optimized again at the B3LYP/6–31G* level. The protein residues around the QM region were subdivided into backbone peptide and side chains moieties. The stabilization effect ΔE_{w(i)} of a given moiety i is then obtained as the change in ΔE (equal to E(Pm) – E(reactant)) when the atomic charges of this moiety are all set to zero (as described in more detail under "Results").

RESULTS

The Reactant and Pm Structures—In the energy-optimized reactant state (see "Materials and Methods"), the Pγ–Oγβ bond length is 1.78 Å (Fig. 2B), which is significantly longer than the distance typical for phosphoanhydride P–O–P bonds (~1.6 Å). This indicates that a certain degree of ground state destabilization is happening in the reactant state, preparing for the dissociation of the γ-phosphophate group. Using the 6–311+G** basis set, we find that the Pm structure is only 2.27 kcal mol^{-1} higher than the reactant structure. Minimum energy path calculations of the catalytic pathway with the conjugate peak refinement method (40) show that this Pm structure is in a local energy minimum and is separated by clear energy barriers from the substrate alone (see "Materials and Methods"). Note that although the three oxygens bonded to Pγ and the "apical" oxygens Oβγ and OWa are co-linear with the Pγ (angle = 178.3°), this is not a bipyramidal structure as would be formed in an associative transition state, because Oβγ and OWa have different distances from the Pγ (2.94 and 1.95 Å, as mentioned above) (25). The three oxygens bonded to Pγ remain nearly stationary during the dissociation, and the difference between the ATP and Pm substrates is mostly due to a small motion (1.1 Å) of the γ-phosphorus atom when it becomes coplanar with its three oxygens (see Fig. 2B). The Mg^{2+} ion is hardly moving and remains hexacoordinated. There are no significant motions in the surrounding protein.

Interactions Stabilizing the Pm State—The "substrate" is defined hereafter as the atoms of the methyl-triphosphate moiety ([PO_4]_3OCH_3\^+, the Wγ and Wm water, and the Mg^{2+} and its four coordinating groups (waters Wγ and Wm, alcohol groups of Thr-186 and Ser-237), as shown in Fig. 2B. For all calculations of interaction energies, the substrate was treated quantum mechanically at the RB3LYP/631G* level. To estimate how much the protein stabilizes the Pm state, the energy difference between the Pm and the reactant structures was computed for the substrate alone (i.e., by removing all other atoms of the system). This energy difference is 44.3 kcal mol^{-1}. In other words, the presence of the protein reduces the energy difference between the reactant and the Pm structures by 44.3 – 2.9 = 41.4 kcal mol^{-1}. This is an astonishingly large stabilization effect, considering that the coordinates of the reactant and the Pm states are so similar (as mentioned above).

In order to understand how this stabilization is achieved, the stabilizing contribution from each protein moiety (breaking the protein into backbone peptides and side chains) was analyzed. The electrostatic interaction energy of a moiety in the Pm state is compared with its interaction energy in the reactant state. Because the substrate is treated quantum mechanically, the electrostatic effect due to the atomic partial

FIGURE 1. Associative versus dissociative mechanisms. The formal charges on the phosphate groups are shown. A, in the "associative" mechanism, the nucleophilic attack of an OH\^− ion (hydrolyzed water) on the tetrahedral γ-phosphophate of ATP\^4− is concerted with Pγ–Oγβ bond cleavage. B, in the dissociative mechanism, an intermediate with a planar PγOβ results from the Pγ–Oγβ bond cleavage occurring before the attack by the hydrolyzed water (arrow).

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charges of a given protein moiety (numbered \(i\)) is obtained here by setting these partial charges to zero and comparing the resulting total electrostatic energy to the corresponding energy obtained with standard (i.e. non-zeroed) partial charges. This yields Equation 1,

\[
\Delta E_{\text{all}}(i) = E_{\text{all}}(\text{normal charges}) - E_{\text{all}}(\text{moiety } i \text{ charges } = 0)
\]

(Eq. 1)

where the “all” subscript indicates that all electrostatic interactions are included (i.e. interactions of moiety \(i\) with both the substrate and the rest of the system). Doing this for both the reactant and the \(P_m\) state structures gives \(\Delta E_{\text{all}}(i)_{\text{Reactant}}\) and \(\Delta E_{\text{all}}(i)_{P_m}\), respectively. The stabilization effect due to group \(i\) is then obtained as follows.

\[
\Delta \Delta E_{\text{all}}(i) = \Delta E_{\text{all}}(i)_{P_m} - \Delta E_{\text{all}}(i)_{\text{Reactant}}
\]

(Eq. 2)

This was done for each backbone peptide group within 14 Å of the triphosphate. A given peptide moiety \(i\) is composed of the atoms [C,O] of residue \(i\) and [N,H,C\(_n\)] of residue \(i + 1\). Such a peptide group has a total net charge of zero (the partial charges of a peptide group are shown in Fig. 3. The resulting stabilization values, \(\Delta \Delta E_{\text{all}}(\text{pep})_i\), are plotted in Fig. 4A (only peptide groups with a significant contribution are shown). This clearly indicates which backbone peptide groups dominantly contribute to stabilizing the \(P_m\) state: peptide 237/238 on the Switch-1 loop (\(-13.2\) kcal mol\(^{-1}\)) and peptides 457/458 on the Switch-2 loop (\(-3.9\) kcal mol\(^{-1}\)). Interestingly, these are all peptides that form H-bonds with either the \(W_{\alpha}\) the \(W_{\beta}\), or the \(\alpha\)- and \(\beta\)-phosphate groups (see Fig. 2B). Because these peptides all directly interact with the substrate, we examined how much of the stabilization in Fig. 4A is due to interactions between each peptide group and the substrate itself (i.e. excluding the electrostatic interactions of the peptide with the rest of the system). This is described below.

**Backbone Peptide Groups That Favor the \(P_m\) Substrate**—The stabilization of the \(P_m\) state from interactions between a peptide group \(i\) and the substrate, \(\Delta \Delta E_{\text{sub}}(\text{pep})_i\), is calculated as described above for \(\Delta \Delta E_{\text{all}}(\text{pep})_i\), except that \(E_{\text{sub}}\) is used instead of \(E_{\text{all}}\). \(E_{\text{sub}}\) is the quantum mechanical energy of the substrate feeling the electrostatic field of the atomic partial charges of the classically treated protein. The resulting \(\Delta \Delta E_{\text{sub}}(\text{pep})_i\) values are plotted in Fig. 4B. Overall, Fig. 4B much resembles Fig. 4A, which confirms that the stabilization effect from the peptide groups is mainly due to their direct interaction with the substrate.

Prominent among the strongly stabilizing backbone peptides in Fig. 4B are six NH groups continuously located on the P-loop, starting from the NH of Gly-182 to the NH of Gly-187. All six act as donors in H-bonds with perfect geometry to the oxygens of the \(\alpha\)- and \(\beta\)-phosphates, both in the reactant (Fig. 2A) and in the \(P_m\) state (Fig. 8). Their stabilizing effect on the \(P_m\) state is explained by the transfer of one negative charge from the \(\gamma\)-phosphate in the reactant state (Fig. 1A) to the \(\alpha\)-\(\beta\)-diphosphate in the \(P_m\) state (Fig. 1B). This charge increase on the \(\alpha\)-\(\beta\)-diphosphate (from approximately \(-2\) to \(-3\)) allows for stronger H-bonds with the above mentioned backbone peptides in the \(P_m\) state of the substrate. Note that Fig. 1B provides only a formal view of the charge distribution. In reality, the transferred charge does not sit only on the oxygen O\(_{\beta}\), but is...
delocalized over the whole α-β-diphosphate group (e.g. see Fig. 5). Peptide 181/182 contributes the most stabilization (−8.2 kcal mol\(^{-1}\)) (see Fig. 4B and Table 1) because the H-bond distance of O\(_\beta\gamma\) to the NH of Gly-182 shortens by 0.2 Å in the P\(_m\) state. The other five H-bonds between the P-loop and the α-β-diphosphate contribute stabilizations ranging from −2.4 kcal mol\(^{-1}\) to −6.5 kcal mol\(^{-1}\) (Fig. 4B), for a total of −26.6 kcal mol\(^{-1}\) (Table 1).

Besides the P-loop, two other peptide groups make large contributions to stabilize the P\(_m\) substrate. Most prominent in Fig. 4B is peptide group 237/238 (on loop Switch-1), which contributes −12 kcal mol\(^{-1}\) stabilization (Table 1). Its Ser-237 C=O carbonyl accepts an H-bond from water W\(_h\) (Fig. 2A), thereby polarizing this water molecule (i.e. increasing the electron density on its oxygen), thus helping to make the oxygen of water W\(_a\) a stronger nucleophile. This reinforces the hydrate complex between W\(_a\) and the P\(_m\)O\(_3\), leading to the unusually close distance between the oxygen and phosphorus atoms (1.95 Å; see Fig. 2B). Finally, peptide 457/458 (on loop Switch-2) has its carbonyl C=O of Gly-457 accepting an H-bond from water Wh, which is itself H-bonded to W\(_h\) (Fig. 2A). The H-bond distance between the oxygen of W\(_h\) and the hydrogen of W\(_a\) goes from normal (1.75 Å) in the reactant state to very close (1.5 Å) in the P\(_m\) state (Fig. 2B). This indicates a cooperative polarization of waters W\(_a\) and W\(_h\) as water W\(_h\) relays the polarization effect from peptide group 457/458 to water W\(_a\). In this way, peptide 457/458 contributes to further stabilizing (by −3.9 kcal mol\(^{-1}\); Table 1) the W\(_a\)P\(_m\)O\(_3\) hydrate complex.

The only backbone peptide group that has a clearly destabilizing effect on the P\(_m\) substrate is peptide 236/237 (−4 kcal mol\(^{-1}\); Fig. 4B). This is due to the fact that its NH of Ser-237 donates an H-bond to the γ-phosphate (Fig. 2A). This H-bond...
becomes weakened when the negative charge shifts away from the $\gamma$-phosphate (and toward the $\alpha\beta$-diphosphate) in the $P_m$ state. Thus, a self-consistent picture of stabilization is emerging, in which the question of whether a protein group is either favoring or disfavoring the $P_m$ state can be explained in terms of its interaction with either the $\alpha\beta$-diphosphate or the $\gamma$-phosphate groups (and the charge shift between the two).

**Side Chains That Stabilize the $P_m$ Substrate**—The total electrostatic interaction of a given side chain $i$ with the rest of the system, $\Delta E_{\text{all}}(\text{side}_i)$, was computed in the same way as described above for the peptide moieties ($\Delta E_{\text{all}}(\text{pep}_i)$). The result is shown in Fig. 6A for the residues making significant contributions. The corresponding side chain contributions from interactions with only the substrate, $\Delta E_{\text{substr}}(\text{side}_i)$, were computed as described for the peptide ($\Delta E_{\text{substr}}(\text{pep}_i)$) and are plotted in Fig. 6B. Comparing Figs. 6A and 6B shows that the dominant stabilizations are again mostly due to direct interactions with the substrate, as observed above for the peptide groups.

By far the strongest stabilization comes from the side chain of Lys-185$^+$ (Fig. 6B), $\Delta E_{\text{substr}}(\text{Lys-185}) = -18.9$ kcal mol$^{-1}$ (Table 1). This is somewhat surprising at first, because its positively charged $-\text{NH}_3^+$ group makes H-bonds with both the $\gamma$- and $\beta$-phosphates (oxygens $O_{\gamma2}$ and $O_{\beta1}$; see Fig. 1 for the atomic nomenclature) with the same perfect geometry (2.6 and 2.7 Å, respectively) in both the reactant (Fig. 2A) and the $P_m$ states. Therefore, a charge transfer from the $\gamma$- to $\beta$-phosphates would seem to have little effect on the interaction with this side chain. However, in the reactant, the distance of its ammonium nitrogen atom to the other atoms of the $\beta$-phosphate group is much closer (3.3–3.6 Å; see Table 3) than to the other atoms of the $\gamma$-phosphate (3.6–4.9 Å). In the $P_m$ state, this difference in distance is even more pronounced (3.2–3.3 Å to the $\beta$-phosphate atoms, 3.8–4.8 Å to the $\gamma$-phosphate atoms). Therefore, and because the negative charge density on the $\beta$-phosphate is distributed over all of its atoms (e.g. see Fig. 5), the increase of this negative charge allows the positive $-\text{NH}_3^+$ group to interact much more favorably with the phosphates in the $P_m$ than in the reactant substrate. This is shown in Fig. 7, which plots the contributions from the electrostatic interaction between the Lys-185$^+$ side chain and the moieties of the substrate. For this purpose, classical partial charges were assigned to the atoms of the substrate. In this charge distribution (Fig. 5), a 0.6 e charge is shifted from the $\gamma$- to the $\beta$-phosphate when going from the ATP (Fig. 5A) to the ADP-P$\gamma$ (Fig. 5B) states. Fig. 7 clearly shows that the electrostatic interaction with the $\gamma$-phosphate is much less favorable in the $P_m$ than in the reactant (ATP) substrate ($+53.4$ kcal mol$^{-1}$), whereas the interaction with the $\beta$-phosphate stabilizes the $P_m$ substrate by $-67.1$ kcal mol$^{-1}$. In sum, this results in the net stabilizing effect of Lys-185$^+$. Obviously, the interaction energies plotted in Fig. 7 are only indicative, because the classical charge distribution used in Fig. 5 is approximate and does not account for electronic polarization effects. Nevertheless, their relative amplitudes are meaningful. Indeed, the sum of the classical interactions in Fig. 7 is $-15.6$ kcal mol$^{-1}$, which is comparable with the quantum interaction with Lys-185$^+$ of $-18.9$ kcal mol$^{-1}$ (mentioned above), showing that the charges in Fig. 5 capture the essence of the charge shift from the $\gamma$- to the $\beta$-phosphates.

Another side chain that exploits the $\gamma$-to-$\beta$ charge shift is Asn-233. It makes a rather poor H-bond (3.1 Å) with oxygen $O_{\gamma2}$ in the reactant (Fig. 2A), which breaks in the $P_m$ state to form a perfect H-bond (2.8 Å) with oxygen $O_{\beta1}$ (Fig. 8). This contributes $-2.9$ kcal mol$^{-1}$ of stabilization (Fig. 6B and Table 1) of the substrate in the $P_m$ state. Ser-236 is the last side chain that makes significant stabilization interactions with the substrate (Fig. 6B). In the reactant state, it donates an H-bond to water $W_{\gamma}$ (the $O_{\gamma2}\cdots OW_{\gamma}$ distance is 2.6 Å), which breaks in the $P_m$ state to form an H-bond to oxygen $O_{\alpha1}$ of the $\gamma$-phosphate (Fig. 8). This switch from a neutral (water) to a charged (PO$_4^-$) H-bond acceptor contributes $-3.4$ kcal mol$^{-1}$ stabilizing interaction with the substrate (Table 1).

The salt bridge between Arg-238$^+$ (on Switch-1) and Glu-459$^-$ (on Switch-2) is known to be essential for the catalytic activity of myosin (41, 42). However, Fig. 6A shows that its role in stabilizing the $P_m$ state is negligible. This is due in part to the fact that the electrostatic interactions of these two side chains with the substrate compete with each other; the $\gamma$- and $\beta$-phosphate moieties are proximal and distal, respectively, to the positively charged Arg-238$^+$ side chain. The favorable interactions with the proximal $\gamma$-phosphate in the reactant state become less favorable ($\Delta E_{\text{substr}}(\text{Arg-238}) = +24.1$ kcal mol$^{-1}$) as the negative charge shifts toward the distal $\beta$-phosphate. The opposite effect occurs for the negatively charged carboxyl group of the Glu-459$^-$ side chain; the unfavorable interaction of the $-\text{COO}^-$ group with the proximal and doubly negative $-\text{PO}_4^-$ becomes less unfavorable as the negative charge shifts further away onto the distal $\beta$-phosphate group, thus favoring the $P_m$ state of the substrate ($\Delta E_{\text{substr}}(\text{Arg-238}) = -13.5$ kcal mol$^{-1}$). In terms of the total electrostatic energy, the stabilizing contribution from Glu-459$^-$ ($\Delta E_{\text{all}}(\text{Glu-459}) = -9.5$ kcal mol$^{-1}$) and the destabilizing contribution from Arg-238$^+$...


**FIGURE 6.** Electrostatic stabilization of the $P_m$ state by side chains (in kcal mol$^{-1}$). This figure is the same as Fig. 4 but for the interactions with a given side chain of residue $i$ (rather than peptide). The contributions from the Arg-238 and Glu-459 side chains, which form a salt bridge (see Fig. 2A), have been combined into a single (rightmost) bar. A, stabilization from interactions of side chain $i$ with the whole system, $\Delta \Delta E_{\text{all}}(\text{side}$). B, stabilization from interactions of side chain $i$ with only the substrate, $\Delta \Delta E_{\text{substr}}(\text{side}$).

**TABLE 3**
Distance (in Å) of substrate atoms to the nitrogen atom of the Lys-185 side chain

| Substrate moiety | Atoms | Reactant state | $P_m$ state |
|------------------|-------|----------------|-------------|
| γ-Phosphate      | O$_{\gamma}$ | 2.59           | 2.66        |
|                  | O$_{\alpha}$ | 4.23           | 4.01        |
|                  | O$_{\delta}$ | 4.93           | 4.81        |
|                  | P$_{\gamma}$ | 3.63           | 3.75        |
| β-phosphate      | O$_{\beta}$ | 2.68           | 2.69        |
|                  | O$_{\beta'}$ | 3.55           | 3.30        |
|                  | O$_{\beta''}$ | 3.62          | 3.35        |
|                  | P$_{\alpha}$ | 3.34           | 3.20        |

$\Delta \Delta E_{\text{all}}(\text{Arg-238}) = 10.6$ kcal mol$^{-1}$ practically cancel out. Nevertheless, the carboxyl -COO$^-$ of Glu-459 makes a strong H-bond to water $W_h$ (Fig. 2A), thereby reinforcing the cooperative polarization of $W_b$ and $W_a$ described above and thus somewhat contributing to the stabilization of the $P_m$ state.

**DISCUSSION**

We have presented here a comparison of the energetics of the reactant (ATP) and the $P_m$ states of myosin using combined quantum/classical methods. In the $P_m$ state, the $O_p$-$P_b$ bond of ATP is broken, and the resulting quasiplanar $PO_3^-$ group forms a hydrate complex with the water that will attack the $P_O_3^-$ in the subsequent step of water hydrolysis. The structure of the protein is very similar in the $P_m$ and the reactant states. The energy difference between the two structures is between 2.3 and 2.7 kcal mol$^{-1}$ (depending upon the basis set; Table 4). This is even more stable than the 8.1 kcal mol$^{-1}$ that had been found before for the $P_m$ state (16, 17). The lower value is probably due to the fact that the strong polarization of all peptide groups in contact with the triphosphate was included here explicitly (Table 2), which results in stronger interactions and therefore in more stabilization. For the same reason, the $P_m$ state is separated here from other states along the hydrolysis reaction by higher barriers than had been found before, where the $P_m$ state had been identified as transition state-like (16, 17).

When the protein is removed, the energy of the $P_m$ substrate is 44.3 kcal mol$^{-1}$ above that of ATP. This shows that an extraordinary stabilization of the substrate in the $P_m$ state is achieved by interactions with the surrounding protein. The electrostatic interactions between the individual protein groups and the substrate were computed to find which protein groups contribute toward the $P_m$ stabilization. This has
revealed that the protein combines two complementary strategies. 1) The protein exploits the shift of negative charge from the γ-phosphate group to the α-β-diphosphate moiety, as illustrated in Figs. 1 and 5. It does so by placing positively charged groups (Lys-185) + and H-bond donors (peptide groups on the P-loop backbone and the Asn-233 side chain) that preferentially interact with the α- and β-phosphate groups, thereby favoring the charge shift. Approximately 75% of the overall stabilization is achieved in this way (Table 1). 2) The other strategy, accounting for the remaining 25% of stabilization, consists in reinforcing the hydrate complex of the PγO−5 with water Wα. To do this, the protein highly polarizes water Wα so that water oxygen OWA bears a higher electron density. This allows OWA to approach very closely to the electrophilic phosphorus atom of the PγO−5 (1.95 Å length; Fig. 2B). Two backbone peptide groups achieve this polarization, either via a direct H-bond to water Wα (C=O of Ser-237) or indirectly via water Wα (itself polarized by an H-bond to C=O of Gly-457). Note that this polarization of water Wα has the added advantage of preparing the breakup of water Wα during the subsequent hydrolysis step (i.e. the generation of the attacking OH− hydroxyl).

**Protein Groups Participating in Stabilizing Strategy 1**—The group that provides the single largest stabilizing contribution (25%) is the Lys-185+ side chain (Table 1). As explained above, the -NH3+ group of Lys-185 is globally closer to the β- than the γ-phosphate group (Table 3), so that it interacts more favorably upon the shift of negative charge from γ to β. It is known that the K185Q point mutation inhibits ATP hydrolysis in myosin...
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(43, 44). In the crystal structure of the human Ras-RasGAP GTase (45), a lysine (Lys-16) has its -NH₃⁺ group at exactly the same position relative to the phosphate groups, so that it can promote the dissociation of the metaphosphate in the same way as described here for myosin. In the structure of F₁-ATPase, the -NH₃⁺ of Lys-162 is located in an exactly equivalent position (46).

The next largest contribution comes from the backbone peptide 181/182 (located on the P-loop). This is not surprising, because the NH group of Gly-182 is making an H-bond with the oxygen O₂ of ATP (i.e. the atom right next to the P-O₂⁻ bond that breaks upon formation of the metaphosphate). This H-bond is maintained in the Pₚ state, but its length shortens from 2.9 to 2.7 Å (O₂ to N₁₈₂ distance), making this interaction stronger in the Pₚ state. Note that the stabilization effect of peptide 181/182 is stronger than indicated by the purely coulombic interaction energy of −8.2 kcal mol⁻¹ (Table 1). This becomes apparent when this peptide is treated quantum mechanically instead of classically, which further lowers the energy of the Pₚ state by 5.08 kcal mol⁻¹ (Table 2). This effect could not be reproduced by upscaling the classical charges of other peptide groups to account for their polarization; see “Materials and Methods”).

This means that this quantum effect is due not only to a polarization of the peptide group itself but also to some degree of charge redistribution on the substrate. Therefore, Gly-182 emerges as one of the residues crucial in stabilizing the Pₚ state. Altogether, its contribution can be estimated to be around 17%. Exactly the same H-bond between a backbone peptide and O₂ is found in the structure of other NTPases (e.g. in Ras-RasGAP with Gly-13 (45) and in the F₁-ATPase with Gly-159 of the β₈ chain (46)).

Five more peptide groups of the P-loop (from residue 183 to 187) together contribute 35% (Table 1) of stabilization. They all make H-bonds to the α- and β-phosphate groups, thus directly favoring the charge shift. The P-loop forms a cradle around the α-β-diphosphate moiety (Fig. 2A). In total, eight H-bonds exist between NH groups of the P-loop backbone and the oxygen atoms of the α- and β-phosphates (Fig. 8). The corresponding residues in the Ras-RasGAP structure are at positions 14–18 (45), and the corresponding residues in F₁-ATPase are 160–164 (46). Their backbone peptides all make exactly the same H-bonds with the α-β-diphosphate as described here for residues 183–187 in myosin. Thus, the P-loop is clearly designed to pull electronic charge from the γ- to the α-β-groups, thereby promoting the dissociation of the metaphosphate. This explains why the P-loop is a characteristic structural feature in other NTPases, where it serves not only as a binding motif (47) but, more importantly, as a device to stabilize the Pₚ state.

Finally, the peptide side chain of Asn-233 is positioned so that it can make an optimal H-bond with O₂ when this oxygen becomes accessible after the Pₐ₂-O₂⁻ bond has broken (Fig. 8). This contributes 4% of the total stabilization (Table 1). Experiments have shown that point mutations of Asn-233 affect the ATPase activity (43). In the Ras-RasGAP GTase, Arg-789 is making exactly the same interaction with the oxygen O₂ of GTP (45). The corresponding interaction is found in F₁-ATPase with Arg-373 (46). Note that several of the peptide groups mentioned above are highly polarized (see Table 2) by their interaction with the strongly charged triphosphate, thereby augmenting their stabilizing contribution. This effect was included here to account for the full amount of stabilization (see “Materials and Methods”).

Protein Groups Participating in Stabilization Strategy 2—Of the groups that reinforce the polarization of water Wₚ, peptide 237/238 contributes 16% of the overall stabilization. The C=O group of Ser-237 on Switch-2 forms an H-bond with the hydrogen H of water Wₚ (Fig. 2A). This H-bond polarizes one of the two O–H bonds of the water (Oₘₚ-H₂ O) (Fig. 2B). The corresponding backbone interaction with the attacking water in Ras-RasGAP is with the C=O of Thr-35 (45), and with the C=O of Ser-344 in the F₁-ATPase (46).

The protein indirectly achieves the polarization of the other bond in water Wₚ (Oₘₚ-H₂ O) by an H-bond with water Wₙ itself polarized by an H-bond with the C=O of Gly-457 (Fig. 2A). This contributes 5% of the overall stabilization (Table 1). The -COO⁻ group of Glu-459 reinforces this effect by also making an H-bond to water Wₙ (Fig. 2A). As a result of having both of its hydrogens engaged in H-bonds, water Wₖ is highly polarized and can form a stronger hydrate complex with the PₚO₅⁻. Finally, this complex is favored by the side chain of Ser-236, which is forming an H-bond with the PₚO₅⁻ that is not formed in the reactant state, contributing a further 4% stabilization.

The Metaphosphate Is Central to the Catalytic Strategy—Pₚ state stabilization is essential to allow a dissociative mechanism, which in turn is the key to lowering the barrier of the ATP hydrolysis reaction. The uncatalyzed reaction is so slow in physiological conditions because the tetrahedral and doubly negative -PₚO₅⁻ group is a very poor target for attack by an OH⁻ hydroxyl. Moreover, the generation of this OH⁻ by hydrolysis of a water molecule is also a slow process. An NTPase like myosin accelerates the reaction by using a dual strategy: 1) promoting the P–O bond dissociation that leads to formation of a PO₃⁻ and 2) deprotonation of the attacking water molecule by a nearby base to form the OH⁻ group. The PₚO₅⁻ is a much better target for attack by the OH⁻ hydroxyl group as compared with the PₚO₂⁻ of ATP, because its planar geometry facilitates access to the phosphorus atoms (compared with the tetrahedral PₚO₂⁻), and it has one less negative charge. To stabilize the Pₚ state, the protein exploits the difference in charge distribution between the ATP and Pₚ substrates, with approximately one negative charge shifting from the γ- to the β-phosphate. The protein groups involved in these stabilizing interactions are mainly the Lys-185⁻ side chain and the backbone NH groups of the P-loop (in particular Gly-182), the Ser-237 C=O moiety, and the Asn-233 side chain (both on the Switch-1 loop). Their relative contributions to the stabilization are listed in Table 1. Equivalent interactions are found in most other NTPases, indicating that the stabilizing mechanism and the catalytic strategy described here are used by NTPases in general.

Reversibility of ATP Hydrolysis in Myosin—Isotope exchange experiments have shown that the oxygen atoms of the terminal γ-PO₃⁻ of ATP and the attacking water molecule can exchange during myosin-catalyzed ATP hydrolysis (48–50). This implies...
that, in the protein, hydrolytic cleavage of ATP is reversible (51, 52). A rapid equilibrium is established between the unhydro-
lyzed myosin-ATP state and the hydrolyzed myosin-ADP·P\textsubscript{i} state, resulting in a reversible cycle that consists of three steps (53, 54): 1) the forward hydrolytic step during which the oxygen of the attacking water is incorporated into the dissociated P\textsubscript{i} 2) P\textsubscript{γ} rotation, during which the phosphate oxygen atoms inter-
change their positions, and 3) reverse hydrolysis during which the reaction proceeds backwards, followed by water release. In
each cycle, one solvent oxygen atom is incorporated into the
γ-phosphate of ATP and one oxygen atom of P\textsubscript{γ} is released as part of a water molecule (48–50). After several cycles, ATP undergoes almost complete oxygen exchange with the solvent. This oxygen exchange is also observed in the presence of actin (49) but at a slower rate (55, 56), because instead of favoring
phosphate rotation, the acto-myosin complex induces product
release. The rates and equilibrium constants of the forward and
reverse ATP hydrolysis reaction in Dictyostelium myosin II at
varying temperatures have been reported (57). The reaction gives a forward rate of 100 ± 10/s, and an equilibrium constant of
13–79 at 293 K, which correspond to a free energy barrier of
14.4 kcal/mol and a reaction free energy of −1.5 to −2.6 kcal/
mol. The metaphosphate state presented here is fully compat-
ible with this reversibility of ATP hydrolysis in myosin, both
structurally and energetically. In terms of the structural path-
way, the dissociative as well as the associative mechanisms
allow for the formation of an ADP/P\textsubscript{i} product in which the P
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