Introduction—Elements of Fundamental Biology

My inquiry on this subject came about when one of my scientific colleague asked me the question, why sulfonation as opposed to phosphorylation? This was back in 1993 while not much was known about the mammalian PAPS relative to ATP. I felt this question would be relevant at a point when more information is gathered on the mammalian, especially when human PAPS biosynthetic enzymes and the sulfur metabolism genes (cDNA) are cloned, expressed and well characterized. Now with the availability of human genome sequence, crystallographic information on sulfo and phospho transferase enzyme(s) and with the knowledge on the phosphate and sulfate metabolism one could ponder and try to understand the biological and evolutionary significance of these two related biochemical processes.

Comparison of Phosphate and Sulfate Metabolism

Phosphate transport

The element phosphorus (P) belongs to group number 15 (atomic weight 30) and in the oxygenated state it is referred to as phosphate (PO$_4^{3-}$) and its acidic version is referred to as phosphoric acid (H$_3$PO$_4$). In higher organisms phosphorus element is obtained in the form of inorganic phosphate for e.g., Ca(PO$_4$)$_2$, trisodium phosphate (Na$_3$PO$_4$), etc. There are many mechanisms by which inorganic phosphate can be transported from the extracellular milieu to the intracellular regions via specific carrier proteins. One of the mechanisms involve either a sodium or proton dependent transport [1]. Regardless of their cation dependency these transporters have consensus amino acid sequence of “GANDVANA” on the amino and carboxyl end. The aspartic residues of the human phosphate transporter are very crucial in binding to inorganic phosphate [1].

Phosphate activation

Inorganic phosphate referred to as ortho phosphate (Pi) becomes organo phosphate by combining with glucose in glycolysis to form glucose 6-phosphate that eventually forms ribose 5-phosphate through pentose phosphate pathway. Ribose 5-phosphate is converted to PRPP. PRPP is the phosphoribosyl donor for salvage as well as de novo synthesis of AMP nucleotide. Once AMP is formed the only route to make ADP is through adenylate kinases that are dependent on ATP. This raises a serious question as to how ATP would be formed from ADP in a tissue that lacks preexisting ATP? Tissues those are rich in mitochondria forms ATP from ADP and Pi through ATP synthase (Fo/Fi ATPase). However, a tissue that lacks mitochondria must rely on ATP independent AMP kinase/adenylate kinase. Regardless of mitochondrial or non/a-mitochondrial cells: preexisting intracellular ADP and ATP are expected to be present for interconversions. However, here I hypothesize that in primordial cell/differentiating cells once adenosine and phosphate are taken up by the cell the adenosine kinase would have to make AMP using Pi and adenosine. This adenosine kinase could make ADP and ATP using Pi which would then form the fundamental pools of AMP, ADP and ATP. Once the basic/fundamental pools of mono, di and tri phosphates of adenosine are established the rest of the evolutionary sophistications can be established (Venkatachalam). This proposed area must be investigated further (Venkatachalam).

The enzyme adenosine kinase (EC 2.7.1.20) [2-7] reported earlier is catalytically dependent on ATP and therefore it is possible that this...
particular enzyme could indeed be a salvage pathway enzyme. I feel being a primordial Pi activation reaction there must be a catalytically ATP independent adenosine kinase and/or AMP kinase that must be present in eukaryotic cell that is involved in the de novo synthesis, that is yet to be isolated. The adenosine kinase (EC 2.7.1.20) that is described by Kornberg et al. [8] perhaps uses ATP as an allosteric regulator as opposed to a source of phosphoryl donor to make AMP from adenosine. This aspect is not clear from their assay systems. Nonetheless, the inhibition of adenosine kinase by bisubstrate analogs could mean direct transfer of phosphoryl from ATP to adenosine in the active site to form AMP and ADP [9]. Careful inspection of the amino acid sequences of the adenosine kinase [9] reveals the characteristic ATP binding motif (GxxxxGK), which could mean this enzyme, could use ATP as a phosphoryl donor or could use ATP as an allosteric regulator. From the crystal structure of human adenosine kinase it is proposed that an aspartic residue is involved in the deprotonation of the ribosyl hydroxyl during phosphoryl transfer [7]. The salvage pathway enzyme adenine phosphoribosyl transferase (APRT, EC 2.4.2.7) that transfer adenine onto C-1 of PRPP to make AMP and P Pi had been well described [9-11]. The formation of PRPP also relies on preexisting intracellular ATP.

Here I have proposed a reaction mechanism for a possible catalytically ATP independent de novo adenosine/AMP kinase reaction (Figure 1). Such an enzyme is warranted in the intracellular milieu since it excludes preexisting ADP or ATP in the primordial cell. Therefore, isolation of this putative catalytically ATP independent adenosine/AMP kinase is very crucial in understanding the fundamentals of biology and the evolution of phosphate activation in organisms (work

![Figure 1: Mechanism of AMP formation and structural components of ATP.](image-url)
in progress: Venkatachalam et al.). AMP can then be activated to ADP by combining with ATP catalyzed by adenylyl kinase (EC 2.7.4.3) [12-18]. The formation of ATP itself can occur by de novo mechanisms through oxidative phosphorylation by combining ADP with inorganic phosphate catalyzed by the mitochondrial proton coupled ATP synthase/ATPase system (EC 3.6.3.14) [19-22]. In cells that lack mitochondria for example (erythrocytes) ATP could be formed by ADP kinase that would use soluble NDP kinase to make ATP (poorly identified). Very little light has been shed on this over all context of ATP formation between these cell types.

**Phosphoryl transfer**

ADP has one phosphonohydride bond that could in theory serve as a high-energy reservoir for driving the thermodynamically unfavorable reaction to the direction of product formation. However, the phosphotransferase enzymes that we know thus far use only ATP and not ADP as a phosphoryl donor. In other words the scission that happens between β−γ phosphates of ATP forms the γ-phosphoryl that is eventually transferred to a recipient moiety by the phosphotransferases to form phosphorylated product [23]. Therefore, we observe ADP only as an intermediate during the biosynthesis of ATP or as a bi product of ATP hydrolyzed reactions [24]. In biochemical systems, ATP cleavage at the α−β position results in adenylyl AMP and PPI formation [25,26]. This process happens during adenylylation, PAPS synthesis, etc. [25-27]. Here we see that the whole AMP (adenylate) portion of ATP is further used in the biotransformations [27] and PPI is only a bi product that is cleaved into two inorganic phosphates by the ubiquitous pyrophosphatase enzyme to push the thermodynamically unfavorable reaction in the forward direction. Thus we see that the molecule ATP can be utilized in two independent ways: a) Upon β−γ cleavage, the resulting γ−phosphate can be transferred to a recipient and the resulting ATP itself is a side product [23 b) Upon α−β cleavage of ATP the resulting adenylyl AMP is the useful phosphorylated component and PPI is just a waste product [25-27]. On rare occasion during the course of s-adenosyl methionine (SAM) synthesis, methionine combines with ATP and the adenosyl moiety is transferred to the sulfur of the methionine to form SAM catalyzed by methionine adenosyltransferase (EC 2.5.1.6) [28-30]. The three phosphates in this reaction of (EC 2.5.1.6) are released as Pi and PPI [28-30]. This is only one enzyme that we know of that splits the bond of ATP between ribosyl unit and the α-phosphate. Thus we see that by using ATP in different modes, an additional variety to the biological processes had formed through evolution.

ATP being a primordial compound various enzymes had evolved based on the principles of the variety that are available for binding ATP. For example, the overall binding of ATP could be anti (i.e., in two dimensions adenine facing westward direction and ribosyl triphosphate(s) facing eastward) or syn (both adenine moiety and ribosyl-phosphate(s) facing the same direction). Although not absolute, it seems like many prokaryotic enzymes bind ATP in syn configuration whereas eukaryotic ATP utilizing enzymes that contains P-loop bind ATP in the anti configuration. By tweaking the molecule into shapes some more variations in binding modalities are achieved.

**Sulfate transport**

In contrast to the element P, sulfur belongs to group 16 (atomic weight 32) and in the oxygenated state it is referred to as sulfate (SO$_4^{2-}$) and its acidic version is referred to as sulfuric acid (H$_2$SO$_4$). In higher organisms sulfur element is obtained in the form of inorganic sulfate [for e.g., CaSO$_4$, MgSO$_4$, etc.]. From the extracellular milieu sulfate is transported to the intracellular regions via specific carrier proteins [31]. The human sulfate anion transporter family (sulfate/anion exchanger) is called SLC26. SLC26A1 has 12 transmembrane domains and a cytoplasmic STAS (sulfate transporters and antigamma factor antagonists) domain at the carboxy end [32]. The STAS domain has been predicted to bind NTP based on the β-sheet scaffold that could accommodate NTP [32]. The proteins of the three human diseases diastrophic dysplasia/achondrogenesis type IB (gene DTD that encodes sulfate transporter) [33], Pendred’s syndrome (PDS gene that encodes iodide-chloride transporter) [34] and congenital chloride diarrhea (CLD gene that encodes chloride-NaHCO$_3^-$ exchanger) [35] contains STAS domain similar to bacterial antigamma factor and has once again been predicted to be regulated by NTP (ATP) hydrolysis [32]. In addition a sodium independent sulfate transporter (SLC26A11) from high endothelial venules has been reported to contain the prosite SLC26 sulfate transporter signature (PS01130), the Piam sulfate transporter domain (P00916) and the STAS domain (pf01740) [36].

**Sulfate activation**

Inorganic sulfate becomes organo sulfate by combining with ATP to form adenosine phosphosulfate (APS) catalyzed by ATP sulfurylase (EC 2.7.7.4), inside the cell [25,26,37,38,39]. APS combines with another molecule of ATP to form PAPS catalyzed by the enzyme APS kinase (EC 2.7.7.4), PAPS, is synthesized by the fused gene product referred to as APS synthese in humans (EC 2.7.1.25) [26,37,39] (Figure 2A). Once PAPS is formed intracellularly it cannot permeate to the extracellular regions similar to ATP. PAPS serve as the universal sulfate donor for many sulfotransferase reactions [23]. In addition, in lower organisms APS/PAPS also serves as the activated oxygenated carrier of sulfur to be reduced to sulfide [40]. Sulfide is then used for methionine synthesis in bacteria, plants etc., [41,42] (Figure 2B). De novo synthesis of methionine doesn’t happen in humans making it an essential amino acid that would have to come from the diet. Methionine is converted into odoriferous compound(s) methylthiol in the human oral cavity, catalyzed by the methionine lyase of the gingival bacteria; *Porphyromonas gingivalis* (Venkatachalam et al., accepted) (Figure 2B). The compounds methyl thiol, dimethylsulfide, dimethylsulfide etc., causes the characteristic morning breath.

**Sulfuryl Transfer**

**Comparison of active site of phospho and sulfio transferases binding:** Among the various elements from the periodic table only the oxygenions of group 15 element (P) and group (16) element (S), that is (PO$_4^{3-}$) and (SO$_4^{2-}$) respectively, had been selected during the course of evolution to be incorporated into the biological systems. What if any evolutionary advantages that these two oxygenions have that the organisms were selective, is poorly understood? Or it could simply mean that both P and S are available in equal amounts from earth. As discussed earlier the molecule ATP can bind to the enzyme either in the anti or syn mode. In Figure 3, I have depicted the syn configuration of ATP. In the anti configuration the adenine portion would assume 180° horizontal flip, upon which the ribose cyclic oxygen unit is mostly masked by the indole (nitrogen 9) of adenine. This rule applies to all cofactors that contain the adenine/腺enosyl unit as part of the cofactor moiety. Thus, PAPS can also assume either anti or syn configuration. It appears that from all the known crystal structures of sulfotransferases, PAPS seems to bind in the anti orientation similar to the bacterial ATP binding to its respective active sites and similar to the various P-loop containing eukaryotic kinases [43] (Figure 4A).

I have mostly eliminated the crystal structures that would
contain the bound recipient cosubstrate to avoid the conformational change induced NTP interactions Figures 4A-4E. In the estrogen sulfotransferase (EST) active site that is bound to PAP and vanadate, a closer look of the ribose C-3 to the 5'-β-phosphate (the residues that are within 5Å) distance are Lys48, Thr51, Lys106, His108 and Phe255 [43]. No metal ions were found to be associated with EST around the putative PAP(s) binding region [43]. In protein kinase A (PKA) that is bound to MgATP within 5Å distance residues Phe54, Lys72 and Asp184 were found [44]. Between EST and PKA there seems to be at least two residues (Phe and Lys) that are common in the active site. Human hexokinase type I bound with ATP analog (AMP-PNP) along with its cosubstrate(s) interacts closely with Gln422, Arg425 and Arg426 [45]. In histidine kinase CheA domain P4, that is complexed with ADPCP and manganese, the closest residues of interactions were Asn409 and His413 [46].

In contrast the homoserine kinase, a prototype of GHMP kinase superfamily, seems to bind ADP in syn mode [47]. Homoserine kinase complexed with ADP shows Ala91, Ser98 and Thr183 residues in close proximity [47]. It seems that upon cosubstrate binding the active site interaction would create a dynamic motion of the protein that would result in residues that are poised for catalysis. Histidine kinase seems to share one exact residue His and one similar residue Asn with that of EST and PKA. On the other hand homoserine that is bound to the ADP in the syn conformation seems to be quite different in terms of amino acid residue selection.

In ATP binding proteins the adenine moiety of ATP uses peptide backbone polar interactions that are independent of protein amino acid sequences to interact with the N-1 and N-6 of adenine ring. Instead they seem to use nonspecific hydrophobic interactions [48]. If the adenine binding loop has the same backbone direction “direct” (NH₂-COOH) then the N-1 is hydrogen bonded to the backbone amidate of the third residue of the loop and the amino group of the adenine at the C-6 is hydrogen bonded to the backbone carbonyl of the first residue of the loop (e.g. cAMP protein kinase). In the “reverse” orientation of the protein, the amino group of the adenine at the C-6 interacts with backbone carbonyl of residue 1 and the N-1 is hydrogen bonded to the

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**Figure 2A:** Transport of inorganic sulfate and the biosynthesis of PAPS.
backbone amide of the second residue of the loop. The geometry of the adenine binding through hydrogen bonding with the respective amino acid backbone motifs exactly mimics the adenine base pairing with thymine in DNA and adenine base pairing with uracil in RNA. Thus, the adenine binding motif which includes three-residue loop and an additional hydrophobic residue probably evolved independently on different time as opposed to a more prosaic divergent mechanism [48]. The adenine rings of PAPS as expected seems to be recognized by entirely different sets of amino acid residues, however overall it possesses hydrophobic pi stacking interactions (Trp53) as seen with other proteins using residues like Trp, Tyr and His (Figure not shown).

Next, I have compared the ribosyl unit and the 5'-phosphate/sulfate and the 3'-phosphate and the associated interactions of the amino acid residues. The phosphates (P-P) at the 5' carbon of the ribosyl unit are present in ATP, CoA, PAPS (phospho-sulfate), NAD+, NADP+, and FAD+. Whereas, the phosphate at the 3' end of the ribose is present only in CoA, and PAPS. In NADP+ instead of 3'-phosphate it is phosphate at the 2'C of ribose Figure 3. Using the adenylate moiety containing coenzymes, I have earlier compared the 5'-P-P interactions versus 5'-P-S interactions with its respective active site residues.

Similarly, here below I have compared the interactions of the 3'-phosphate of various coenzymes and its interactions with its active site residues within 5Å distance (Figures 5A-5F). While ATP phosphates are almost always complexed with magnesium the PAPS binding doesn't seem to require metal ions for either binding of the nucleotide substrate or for catalysis. EST at the 3'-phosphate end seems to interact with Arg130, Ser138 and Arg257. The enzyme 3'-phosphonucleotidase also called PAPase (Hal2p) complexed with calcium, magnesium, and reaction substrate PAP interacts with Glu72, Asp142, Asp145, Thr147 and Asp294 [49]. PAPase is an exonuclease that cleaves the 3'-phosphate to form inorganic phosphate (Pi) and AMP. The acidic residues in this case may interact with metal cation and the metal cation can then interact with the negative 3' phosphate group of PAP. The indirect interaction of acidic residues with the 3'-phosphate is very similar to the phosphate transporter protein interactions with phosphate anion that I had described earlier [1]. It is very interesting that in EST since there is no metal binding, the phosphate negative charges are interacted electrostatically by the basic arginine residues (Figure 4A). In RNase A bound to dCP-A mimicking the semi transition state shows the catalytic residues His12, His119 and few closely interacting residues Gln11 and Lys41 [50].

In Oral Cavity Bacteria

The enzyme CAMP 3'-5' phosphodiesterase is very interesting that the phosphate is covalently bonded to 3' and 5'C. The residues that are involved in this case are His234, His238, His274, Asp275 and Asp392 [51]. There are at least two basic residues (Arg and Lys) that are perhaps involved in charge neutralizations. The enzyme cAMP 3'-5' phosphodiesterase is very interesting that the phosphate is covalently bonded to 3'C and 5'C. The residues that are involved in this case are His234, His238, His274, Asp275 and Asp392 [52]. Here it could be speculated that the acidic Asp residues interacts with cation magnesium and the magnesium can then balance the charges of the 3'-phosphate. However the His residues in the 3'-5'-phosphodiesterase could be catalytically involved in a manner.

Figure 2B: Metabolism of sulfite in organisms. In humans sulfate is converted into PAPS. In bacteria PAPS can be further converted into sulfide and eventually into methionine. Methionine is metabolized into methythiol by the oral cavity gingival bacteria Porphyromonas gingivalis.
Figure 3: PAPS and other adenylate containing coenzymes.
Figure 4: Comparison of a sulfotransferase with several phosphotransferase enzymes, focusing on the active site. The enzymes are all shown in a ribbon representation (grey) with the cofactor PAP or ATP analogs (colored sticks – thick) in similar orientations (ribose C3* to the beta phosphate superposed). Residues within 5 Å of the beta phosphate are shown as thin sticks in red.

4A: Estrogen sulfotransferase with inactive cofactor PAP and vanadate (PDB ID: 1BO6) [43]. Residues shown are: Lys48, Thr51, Lys106, His108 and Phe255.

4B: Protein Kinase A (PKA) double mutant model of PKB in complex with MgATP. (PDB ID: 1Q24) [44]. A 24 residues substrate polypeptide is shown as a ribbon. Residues shown in color are Phe54, Lys72 and Asp184.

4C: Human hexokinase type 1 complexed with ATP analog AMP-PNP (PDB ID: 1QHA) [45]. In addition to the ATP analog, glucose and glucose-6-phosphate can also be seen in the back of the molecule. Residues highlighted are Gln422, Arg425 and Arg426.

4D: Histidine kinase CheA domain P4 in complex with ADPCP and manganese (PDB ID: 1I5A) [46]. Residues highlighted are Ans409 and His413.

4E: Homoserine kinase complexed with ADP (PDB ID: 1FWK) [47]. Residues highlighted are Ala91, Ser98 and Thr183. Metal ions (Mg in panels B, C, and E; Mn in panel D) are shown as small spheres.
Figure 5: Comparative analysis of a sulfotransferase, with several other enzymes using similar cofactors, focusing on the 3’ phosphate group. The enzymes are all shown in a ribbon representation (grey) with the cofactor PAP or ATP analogs (colored sticks – thick) in similar orientations (ribose rings have been superposed). Residues within 5 Å of the 3’ phosphate (or equivalent) are shown as thin sticks.

5A: Estrogen sulfotransferase with inactive cofactor PAP and vanadate (PDB ID: 1BO6) [43]. Important residues around the 3’ phosphate shown are: Arg130, Ser138 and Arg257.

5B: PAPase Hal2p complexed with calcium, magnesium, and reaction substrate, PAP. (PDB ID: 1KA1) [49]. Residues shown are Glu72, Asp142, Asp145, Thr147 and Asp294.

5C: RNase A complexed with d(CpA). (PDB ID: 1RPG) [50]. Residues highlighted are Gln11, His12, Lys41 and His119.

5D: Aldose reductase, holo form (PDB ID: 1AH4) [51]. Residues highlighted are Lys262, Ser263, Val264, Thr265 and Arg268.

5E: Catalytic domain, of cAMP-specific 3’-5’ cyclic phosphodiesterase 4B complexed with AMP (PDB ID: 1ROR) [52]. Residues highlighted are His234, His238, His274, Asp275 and Asp392.
that is mechanistically very similar to RNase A. In Coenzyme A (CoA) [53] moiety it contains a 3'-phosphate and what exact residues interact if any at 5Å distance among the CoA binding protein is not clear at this point although one would be tempted to think it would be similar to PAP(S) interactions.

Conclusions and Future Directions

The molecule ATP seems to be used in a variety of mechanisms like anti or syn mode of binding. Catalytically, cleavage can happen at the β-γ bond and the phosphoryl group can be transferred to a small molecule or a macromolecule such as proteins. The phosphorylated compound would contain two negative charges. Energetically diions are less reactive (more stable) and upon active site protonation it can be cleaved by specific phosphatas.

Phosphorylation/dephosphorylation, on/off switch in transiently regulating a protein is a universal mechanism of control. ATP when cleaved between α-β bonds generates an adenlyte (AMP) that can be transferred on to a recipient small molecule sulfuryl to form adenosine 5'-phosphosulfate (APS) or to proteins to form adenylylated protein. Occasionally the whole moiety of adenosine from ATP can be transferred on to sulfur of methionine to form SAM.

From the reports that are available it appears that PAPS bind in an anti mode to sulfootransferases (e.g. EST) much like ATP binding to PKA. The sulfootransferases [54] transfer sulfuryl group from PAPS to the recipient molecule [55,56]. The recipient molecule receives one negative charge and as a monooanion it is less stable (more reactive) and the sulfuryl group is removed by the sulfataises that would require less energy relative to phosphate. The process sulfurylation/de sulfurylation mechanism occurs universally in organisms. Comparison of putative phospho-sulfo bond interaction at the 5'-end seems to have very little global consensus. Although one would be tempted to say that there is at least one basic residue that could be present in most of them (Lys/Arg/His). Interestingly the phosphate at the 3'-C has an Arg residues to interact with the negatively charged phosphates if there are no metal binding requirements, for e.g. EST. If there is metal binding involved then the bound 3'-phosphate is coordinated to metal cation (magnesium) and the positively charged magnesium interacts with negatively charged Asp residues (many phosphotransferases/kinas). If the 3'-phosphate is meant to be catalytically removed, then it seems like there are at least one or two basic (His) residues (e.g. RNAsae, 3'-5'-phosphodiesterase).

In future, I propose to isolate, molecularly clone the cDNA/gene, express and characterize the primordial adenosine kinase in relation to the de novo inorganic phosphate activation in mammals.

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References