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Unexpectedly General Replaceability of ATP in ATP-Requiring Enzymes

Yasunori Kinoshita and Koichi Nishigaki[1]

Department of Functional Materials Science, Saitama University, 255 Shimo-Okubo, Urawa, Saitama 338

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ATP-dependent enzymes were investigated as to the stringency of their ATP requirement. For all the enzymes examined except firefly luciferase (including hexokinase, polynucleotide kinase, T4 DNA ligase, and T4 RNA ligase) ATP could be replaced with dATP, contradicting previous data. Considering the replaceable nucleotides, not only kinases (low stringency as to ATP-requirement) but also other enzymes (moderate stringency) were typed as phosphate-directed ATP recognition. Through this study, an exact view of ATP-requiring enzymes which have a profound influence on the concentration in a cell of ATP, a metabolic and regulative key substance, was obtained, and a technically useful, fluorescent ATP-substitute (2AP-TP) was introduced.

Key words: ATP-binding domain, ATP replaceability, ATP-requiring enzyme, ligase, stringency as to ATP-requirement.

Adenosine triphosphate, a key molecule in biological chemistry, is at the heart of both biological information systems and energy/substrate metabolisms. It functions by transmitting signals as a phosphate donor, synthesizing information biopolymers, generating ion-concentration gradients or mechanical forces, and enabling almost all naturally-unfavorable reactions to occur (*1, 2*). Therefore, the synthesis and utilization of ATP *in vivo* have been vigorously studied. Likewise, ATP-dependent enzymes such as kinases have been the keen object of investigation. In those studies, ATP has often been regarded as a reagent which cannot or can hardly be replaced by another analogue (*3-5*). If we think about the extensive contribution of ATP to basic metabolism, and the strict regulation of the *in vivo* ATP/ADP ratio and the ATP concentration (*6*), whether or not ATP can be replaced by another reagent is crucial for understanding not only enzyme reactions but also the harmonic regulation of molecular systems in cells.

Plenty of ATP-requiring enzymes have been both extensively and intensively studied, and their complicated reaction mechanisms and fine structures have been elucidated. In particular, recent advance in structural studies has provided otherwise unobtainable information. However, little is known systematically about the replaceability of the ATP of these enzymes, which has a profound meaning in molecular systems.

The knowledge so far obtained has allowed us to classify these enzymes into three categories as to ATP requirement: low, moderate and high stringencies. Firstly, most kinases so far investigated fall into the low stringency category, as follows: T4 polynucleotide kinase and yeast nucleoside-diphosphate kinase are known to perform a phosphorylation reaction using either GTP, CTP, UTP, or dATP (*7*); glucokinase from *Propionibacterium shermanii* can utilize inorganic polyphosphates in place of ATP (*8*), and so on. Secondly, numerous enzymes have been reported to exclusively require ATP for their reactions (*i.e.*, high stringency); firefly luciferase (*9, 10*), T4 DNA ligase (*4, 5*), and others. Finally, the remaining ATP-requiring enzymes should, consequently, be assigned to the moderate stringency category. In this category, a variety of enzymes are included, such as kidney Na^+-K^+-ATPase (*11*) and yeast topoisomerase (*12*). We examined these three categories of enzymes and found some novel facts, leading to an important idea: a general feature of phosphate-directed ATP-recognition of ATP-dependent enzymes.

MATERIALS AND METHODS

Reagents—2-Aminopurine riboside triphosphate (2AP-TP) was prepared by the authors following the method of Ward and Reich (*13*), and the other reagents were commercially obtained: ribonucleotide triphosphates and dideoxyadenosine triphosphate from Pharmacia Biotech (Uppsala); and the others, including hypoxanthine/xanthine-riboside triphosphate, etheno-adenosine triphosphate (ε-ATP), 3′-amino, 3′-deoxyadenosine triphosphate (3′-NH_2-ATP), cytosine β-D-arabinofuranoside 5′-triphosphate (araCTP), cordycepin triphosphate (CorTP), adenosine β-D-arabinofuranoside 5′-triphosphate (araATP), and polyphosphates, from Sigma Chemical (St. Louis, USA). The enzymes used here were from commercial sources: PNK,

[1] To whom correspondence should be addressed. Tel: +81-48-858-3533, Fax: +81-48-858-3533

Abbreviations: AAA, ATPases associated with a variety of cellular activities; 2AP-TP, 2-aminopurine riboside triphosphate; araATP, adenine β-D-arabinofuranoside triphosphate; ARS, aminoacyl-tRNA synthetase; ε-ATP, ethenoadenosine triphosphate; BSA, bovine serum albumin; CoA, Coenzyme A; CorTP, 3′-deoxyadenosine triphosphate or cordycepin triphosphate; $GlcNH_2$-6p, glucosamine-6-phosphate; 3′-NH_2ATP, 3′-deoxy,3′-aminoadenosine triphosphate; NTP, either ATP, UTP, GTP, or CTP; $(P)_2$, inorganic pyrophosphate; $(P)_3$, inorganic triphosphate; $(P)_4$, inorganic tetraphosphate; PD, phosphate donor; PEG 6000, polyethylene glycol 6000; PNK, polynucleotide kinase; T4, bacteriophage T4; T7, bacteriophage T7.

TdT, T4 RNA ligase, and T4 DNA ligase from Takara Shuzo (Kyoto), and yeast hexokinase and myosin from rabbit muscle from Sigma Chemical. The oligonucleotides, Oligo-1-Oligo-5, were custom-synthesized by The Midland Certified Reagent (Midland, USA) and Takara Shuzo.

Kinase and ATPase Reactions—Kination reactions were performed using various phosphate donors, as shown in Table I, whereas the phosphate acceptors were Oligo-1 (5′-CTCACTCC-3′) for PNK and glucosamine for yeast hexokinase. The reaction mixtures (10 μl), containing 7 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 μM Oligo-1 (or 1 mM glucosamine), 1 unit/μl PNK (or yeast hexokinase), and 0.1 mM of a phosphate donor, were incubated for 1 h at 37°C.

The hydrolysis reaction with myosin was performed at 37°C for 60 min in the same reaction buffer as described above. The nucleotides were precipitated with acetone and then analysed by thin layer chromatography.

Ligase Reactions—A reaction mixture (8 μl), containing 7 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 10 pmol of bacteriophage M13 single-stranded DNA as a template, 10 pmol of 5′-phosphorylated Oligo-2 (5′-pGCTTGCTTTCGAGGTGAATT-3′), and 10 pmol of Oligo-3 (5′-TTAATTGTATCGGTTTATCA-3′), was heated at 65°C for 5 min, and then gradually cooled to 10°C at 0.05°C/s to anneal the oligonucleotides to the template DNA (the two oligonucleotides were flanked head-to-tail on the template DNA). Ligation was started by the addition of 1 μl of either 1 mM ATP, dATP or 2AP-TP, and 1 μl of T4 DNA ligase (5 units). The reaction mixtures were kept at 37°C for 1 h, and then analysed by electrophoresis and silver-staining (see "*Detection Methods*").

Oligo-4 (5′-GGAGTCTTAc-3′) and Oligo-5 (5′-ATGAA-TTACCTTAAAAAAAAAAAA-3′) were used as the acceptor and donor for ligation, respectively, where the lower case letter, c, stands for a ribonucleotide, the other letters representing deoxyribonucleotides. Prior to the ligation experiments, the donor oligonucleotide was 5′-phosphorylated with PNK and 3′-dideoxyadenosylated with terminal deoxynucleotidyl transferase, and then processed for purification [oligo(dT)cellulose column]. Ligation reactions were performed in 2 μl of a reaction mixture containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 μg/ml BSA, 1 mM hexammine cobalt chloride, 25% PEG 6000, 25 μM of the acceptor, 5 μM of the donor, 5 units/μl T4 RNA ligase, and 0.1 mM of an energy donor for 3 h at 25°C (*14*).

Detection Methods—Reaction products were detected by gel electrophoresis or thin layer chromatography. If not otherwise mentioned, gel electrophoresis was performed with a gel of 15% acrylamide and 8 M urea dissolved in 20 mM Tris-acetate, 10 mM sodium acetate, and 1 mM EDTA (pH 8.0), followed by silver-staining. Silver-staining (*15*) was performed on all specimens including oligo- and mononucleotides, and glucosamine. (Note that the silver-staining of mononucleotides, and sugars was not conventional.) Thin layer chromatography for the detection of myosin hydrolysis was carried out on a Funacel SF cellulose thin layer plate (Funakoshi, Tokyo) with a developing solvent of isobutyric acid/0.5 M ammonium hydroxide [5 : 3 (v/v)]. Spots on a TLC plate appearing on UV irradiation were directly photographed.

RESULTS AND DISCUSSION

Enzymes of Low Stringency as to ATP-Requirement—Hexokinase (yeast) and polynucleotide kinase (T4) were investigated to clarify the nature of their "low stringency," and a bibliographical approach was also made for other kinases (Table I). As to T4 polynucleotide kinase, which catalyzes the transfer of the γ-phosphate of ATP to the 5′-hydroxyl terminal of a nucleic acid, and elimination of the 3′-phosphate of a nucleic acid, we observed that a wide spectrum of nucleotides (including 2AP-TP and ε-ATP) and inorganic polyphosphates could serve as phosphate donors (Fig. 1, A and B). This finding was quite unusual since it means that T4 polynucleotide kinase only requires the triphosphate moiety of a nucleoside triphosphate and that this enzyme can disregard the other molecular parts in the kination reaction. In particular, the fact that inorganic triphosphate can replace ATP is so uncommon that we could only find one other report of such an observation, for glucokinase (*8*), probably meaning a lack of this viewpoint. Interestingly, some studies on chemical evolution gave rise to the idea that in a primordial chemical world, not ATP, but a more primitive molecule such as inorganic triphosphate played the role of the ATP (*16*).

There have been contradictory reports as to whether hexokinase (yeast) can utilize dATP or not (*17, 18*). As shown in Fig. 1C, hexokinase does use dATP under physiological conditions. In addition, other types of nucleoside triphosphates examined (*i.e.*, CTP, GTP, UTP, ITP, dCTP, dGTP, and dTTP) could take the place of ATP (data not shown).

As has been well established, ATP is composed of a triphosphate moiety and a nucleoside one, the latter itself being a complex of a ribose and an adenine base (as shown

TABLE I. **Replaceability of non-ATP nucleotides and triphosphate examined as to kinases and myosin.**[a]

Enzyme (source[b])	Nucleotide tested[c]										Ref.
	dA	G	dG	C	dC	U	dT	I	P_3	Others	
Nucleoside-diphosphate kinase (Sc)	○	○	○	○	○	○	○	—	—		*38*
Phosvitin/casein type II kinase (Hc)	○	○	○	—	—	—	—	—	—		*39*
Thymidine kinase (Sc)	○	—	—	—	—	—	—	—	—		*40*
Adenosine kinase (hl)	○	○	○	—	—	—	—	—	—		*41*
Cyclic AMP-independent glycogen synthase kinase (rsm)	○	—	—	—	—	—	—	—	—		*42*
N-Acetylglucosamine kinase (Ca)	○	—	—	—	○	—	—	—	—		*43*
5′-Phosphate-polyribonucleotide kinase (vv)	○	—	—	—	—	—	—	—	—		*44*
Glucokinase (Ps)	—	—	—	—	—	—	—	—	○		*8*
Hexokinase (Sc)	◉	●	●	●	●	●	●	●	▲		*17, 18*
Polynucleotide kinase (T4)	◉	◉	●	◉	●	◉	◉	◉	●	●[d]	*7*
Myosin (rm)	◉	◉	○	◉	●	◉	○	◉	○	●[e]	*21, 22*

[a]Replaceable: ○, bibliographically known; ●, determined in this study; ◉, both. Not replaceable: ▲, this study. Unknown: –. [b]Sc, *S. cerevisiae*; Hc, HeLa cell; hl, human liver; rsm, rat skeletal muscle; Ca, *Candida albicans*; vv, Vaccinia virus; Ps, *Propionibacterium shermanii*; T4, bacteriophage T4; rm, rabbit muscle. [c]All nucleotides are triphosphated. P_3 denotes inorganic triphosphate. [d]XTP, araATP, ε-rATP and ddATP were replaceable. See the legend to Fig. 1 for the abbreviations. [e]3′-NH_2ATP, araATP and ε-rATP were replaceable.

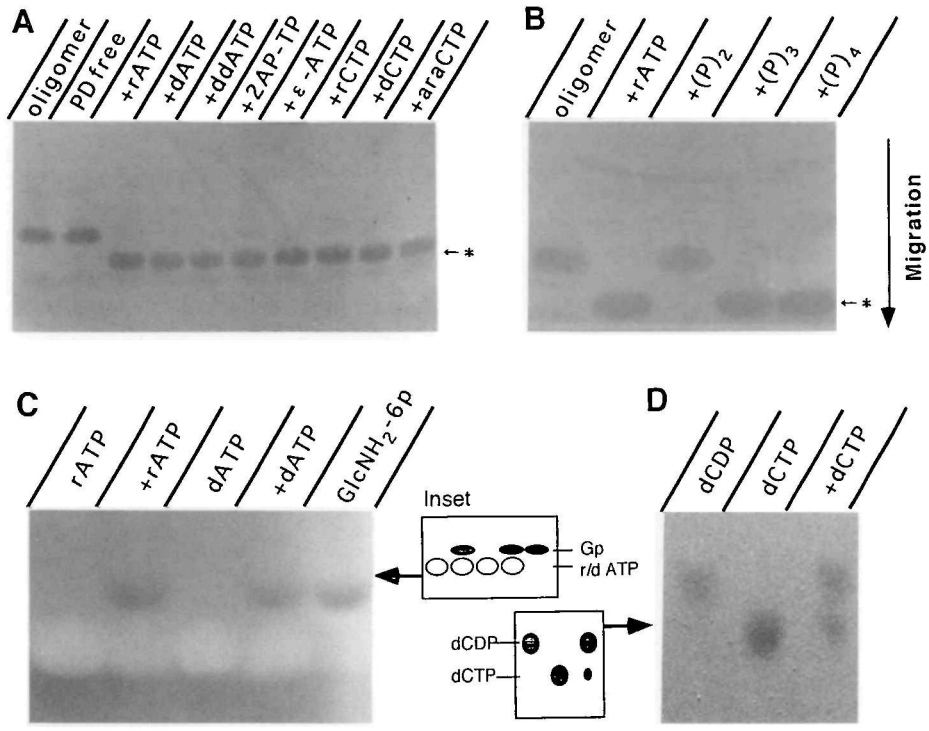

Fig. 1. **Relaxed stringency as to ATP-requirement of T4 polynucleotide kinase, yeast hexokinase, and myosin.** (A and B) ATP-replaceability of possible nucleotides/polyphosphates in the reaction of polynucleotide kinase (PNK). Kination reactions were performed as described under "MATERIALS AND METHODS." Note that the phosphate donors (PDs) used for the kination reactions are indicated above the lanes with a + sign. Gel electrophoresis and silver-staining were performed for detection. Phosphorylated oligomers (*) migrated faster. (C) Phosphorylation of glucosamine by yeast hexokinase. The phosphate donors examined were rATP (+rATP) and dATP (+dATP). The electrophoretic references (rATP, dATP, and $GlcNH_2$-6p) were run in the remaining lanes. A gel of 20% acrylamide containing 20% glycerol but not urea was used for electrophoresis. In this study, conventional silver-staining was found to detect μg-amounts of sugars. Unphosphorylated glucosamines did not migrate into the gel (and so are not observable). For clarity, see the inset ("Gp" denotes $GlcNH_2$-6p). (D) Hydrolysis of dCTP by myosin. The product (+dCTP) and the references (dCDP and dCTP) were detected on a thin layer chromatogram.

in Fig. 3). The triphosphate moiety contains high energy phosphate-to-phosphate bonds. For some kinases, an "in-line mechanism" has been proposed for their reaction (*19, 20*), in which the triphosphate part must be essential for direct reaction as well as molecular recognition. As a result, the contributions of the other moieties of ATP are less significant. Therefore, the low-stringency as to ATP requirement of these enzymes should be a reflection of their indifference to the nucleoside moiety. In Table I, the kinases which have been reported to utilize non-ATP nucleotides are listed. Noticeably, all these examined can uniformly use dATP in place of ATP. Considering that only a few nucleotides were examined in the studies reported, it must be possible to expand the list of replaceable nucleotides for these enzymes, probably to the level of yeast hexokinase. Since a number of kinases are not included in this list, we cannot take the characteristics observed here as general ones for kinases. [Current extensive studies on signal transduction have shed light on a number of kinases such as the *src* family and MAP kinases, but we could not obtain any reliable information on ATP-replaceability as to these kinases (most kinase preparations are contaminated by ATP by chance or by intention).] However, we can obtain a rough image of *low stringency* from the kinases tested: (i) most can utilize dATP and even more species of nucleotides in addition, and (ii) the triphosphate moiety is essential in their reaction, but the nucleoside moiety is not.

Myosin was reported to utilize dATP, dGTP, dTTP, NTPs (*21*), and triphosphate (*22*) in place of ATP (Table I). Most of these nucleotides were confirmed again here to be replaceable and, in addition, dCTP, 3′-NH_2ATP, araATP, and ε-ATP were also found to be replaceable (Fig.1D). Therefore, myosin, which is not a kinase but an ATPase, is also categorized as having low stringency as to ATP requirement. The X-ray structure of the myosin motor domain complexed with a nucleotide revealed that the protein undergoes many interactions with the phosphate moiety but only a few with the nucleoside moiety (*23*). This observation is consistent with the image of *low stringency* described above.

Enzymes of High Stringency as to ATP-Requirement—In some ATP-requiring enzymes, reportedly, ATP cannot be replaced with anything. Among the latter are firefly luciferase (*3*) and T4 DNA ligase (*4, 5*). We re-examined these enzymes, which are apparently of "high stringency." We found that, as shown in Fig. 2A, T4 DNA ligase can utilize not only dATP but also 2-aminopurine nucleoside triphosphate (2AP-TP) in place of ATP. This discrepancy can be rationalized by taking kinetics into consideration (Fig. 2B). Since the dATP-utilizing reaction rate of T4 DNA ligase is one order of magnitude lower than the ATP-utilizing one, experiments involving a short reaction period must fail to reveal positive results as to dATP-utilization (*4, 5*). Considering its reaction mechanism, we prefer to classify this enzyme into the *moderate stringency* category, since it recognizes both the triphosphate and base parts of ATP, which is more complicated than in the case of *low stringency* enzymes. With regard to firefly luciferase, we obtained results (data not shown) supporting the preceding papers (*9, 10*), in which it was reported that it only utilized ATP for light emission. [Firefly luciferase is known to produce diadenosine tetraphosphate from ATP, in the presence of pyrophosphatase (*24*). In this case, ATP can be replaced with adenosine tetraphosphate, dATP, or GTP for formation of the corresponding homo-dinucleotide polyphosphate: diadenosine pentaphosphate, dideoxyadenosine tetraphosphate, and diguanosine tetraphosphate, respectively.] This was confirmed through experiments perform-

ed with a dATP concentration of 5 μM, which is five thousand times higher than the lower detection limit of ATP (*25*). Therefore, this enzyme is truly of *high stringency*. However, there the possibility remains that the high stringency observed for luciferase may be an artifact due to the method used to measure activities, *i.e.*, chemiluminescence intensity, a differential quantity, while the activities of all the other enzymes listed here were measured as an integral quantity (or the cumulative amount). In a recent study, firefly luciferase was found to have a similar, though distinct, functional structure to that of aminoacyl-tRNA synthetase (ARS) (*26*), which is responsible for adenylation at the COOH group of a reactant, as shown in Fig. 3. In addition, a similar transfer reaction from B to C occurs for both enzymes. (This high similarity observed between evolutionarily-distant genes seems to be proof of the divergent evolution of some ATP-requiring enzymes. Thus, aminoacyl-tRNA synthetases are also possible *high stringency* candidates.) This high stringency may be explained by the multiplicity of contacts between ATP and the enzymes required for multiple elementary reaction steps, such as the accomodation of ATP in a proper site, the adenylation reaction and the transfer reaction. Furthermore, although we do not know the exact reason why this case is more stringent than the case of, for example, T4 DNA ligase, we can state that the latter covalently binds AMP, transiently, on its surface and thus firmly fixes it, whereas the former does not and therefore needs the accumulation of weak interactions.

On the other hand, for RNA polymerase, which is another type of *ATP-requiring* enzyme, a teleological explanation for its stringency is more easily acceptable (*27*), because possible contamination by deoxyribonucleotides in RNA synthesis has to be excluded in order to avoid the minglemangle of ribo- and deoxyribonucleotides. In addition, since each nucleotide has a different base moiety, the nucleotide binding site of this enzyme must grasp the remaining common parts, *i.e.* the ribose and phosphates of the nucleotide, which leads to discrimination between ribo- and deoxyribonucleotides, without discrimination between bases. However, Wyatt and Walker found that T7 RNA polymerase incorporates dATP and other deoxyribonucleotides into RNA, though in a non-successive manner and under non-physiological conditions (*28*). Therefore, T7 RNA polymerase is not of high but of moderate stringency.

Enzymes of Moderate Stringency as to ATP-Requirement—The ATP-requiring enzymes which are not assigned as being of either *high* or *low stringency* are logically of *moderate stringency*. In addition to T4 DNA ligase and T7 RNA polymerase discussed above, Na^+-K^+-ATPase (*11*), phosphoribosylpyrophosphate synthetases (*29*), mammalian glutamine synthetase (*30*), acetate:CoA ligase (*31*), and topoisomerases (*12*) belong to this category according to the respective reports. The enzymes of this category usually allow the replacement of ATP with dATP (at least, those mentioned above).

We performed in-depth analysis of T4 RNA ligase, which is another possible candidate for the high stringency category. As shown in Fig. 4A, dATP can replace ATP.

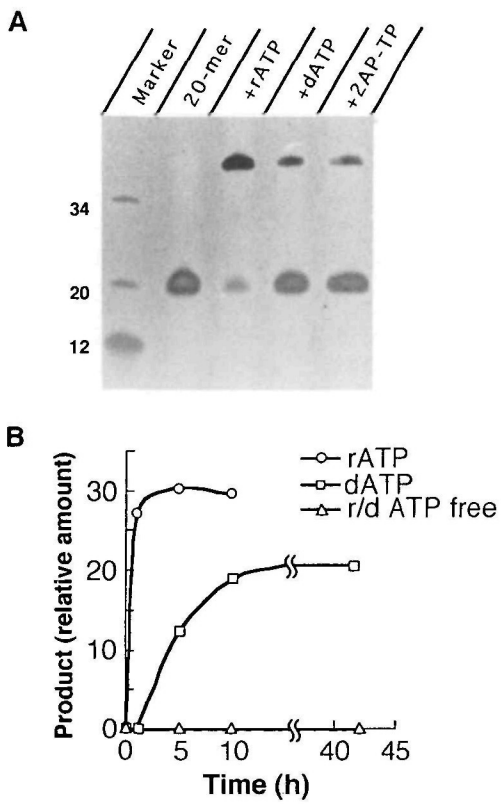

Fig. 2. **Stringency as to ATP-requirement of T4 DNA ligase.** (A) Replaceability of ATP with dATP and 2AP-TP. Two oligodeoxynucleotides, Oligo-2 (20-mer) and Oligo-3 (20-mer), head-to-tail on a single-stranded DNA of bacteriophage M13, were ligated with T4 DNA ligase using an energy donor, as indicated above each lane, and then gel-electrophoresed (see "MATERIALS AND METHODS"). (B) Time course of the ligation reaction. The reaction was performed at 16°C using 1 unit of enzyme under, otherwise, the same conditions as in (A). Densitometry was performed for quantification.

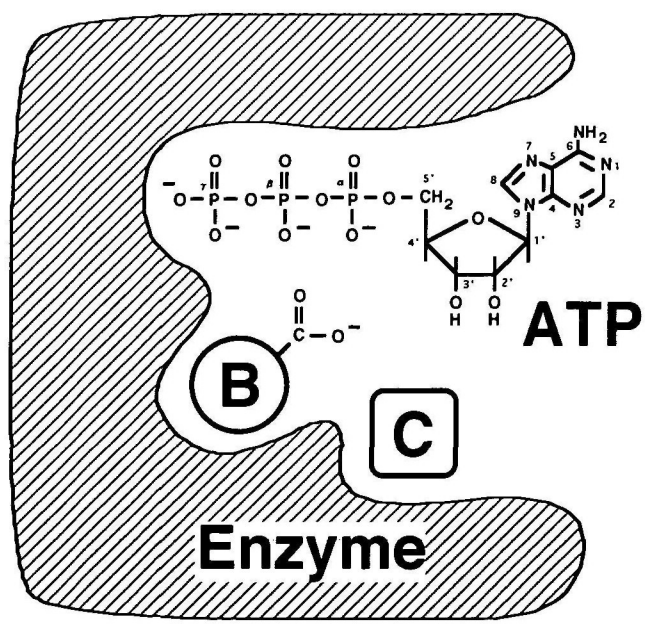

Fig. 3. **Schematic representation of three component reactions postulated for firefly luciferase and aminoacyl tRNA synthetase.** ATP reacts with substrate B, *i.e.*, luciferin for luciferase or an amino acid for ARS, at the reaction center of each enzyme, adenylated substrate B being formed. Then, the transfer of substrate B onto substrate C (*i.e.*, O_2 or tRNA, respectively) occurs.

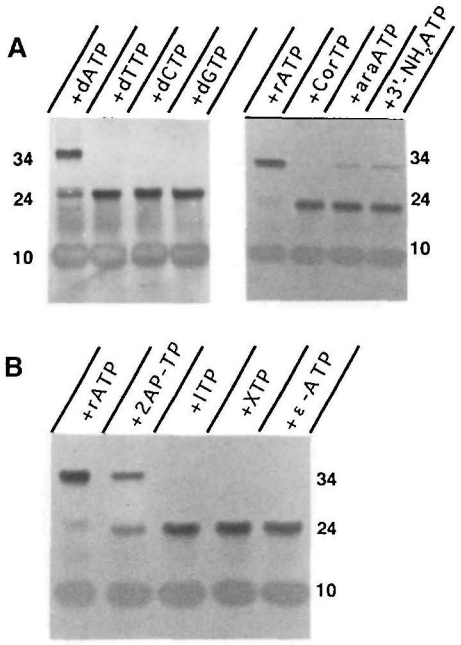

Fig. 4. **Stringency as to ATP requirement of T4 RNA ligase.** Two oligonucleotides, Oligo-4 (10-mer) and Oligo-5 (24-mer), were ligated using an energy donor, as indicated above each lane (for details, see "MATERIALS AND METHODS"). Gel electrophoresis was performed for analysis. (A) Regarding non-ribose sugar nucleotides. (B) Regarding non-adenine base nucleotides.

Moreover, adenosine β-D-arabinofuranoside triphosphate (araATP), 3′-amino,3′-deoxyriboadenosine triphosphate (3′-NH_2ATP), and 3′-deoxyadenosine triphosphate (cordycepin triphosphate) could also be utilized, though the activity was weak (Fig. 4A). However, dideoxyadenosine triphosphate, which has neither OH nor NH_2 at both 2′- and 3′-positions of pentose, was not used, suggesting the importance of the presence of proton-donor(s) at these positions for ATP-like activity. We also examined non-adenine-base-containing nucleotides, although the non-replaceability of CTP, TTP, and GTP had already been reported (*32*). Those containing guanine, thymine, hypoxanthine, xanthine, or etheno-adenine as a base showed no detectable activity, whereas 2-aminopurine nucleoside triphosphate (2AP-TP) showed remarkable activity (Fig. 4B). This may mean that the oxo group, a proton-acceptor, and the bulky group (etheno) at the 6-position of purine are inhibitory. The knowledge thus obtained fairly well reveals the atomic environment of T4 RNA ligase surrounding the substrate, ATP. The whole properties of the nucleotide replaceability of an enzyme can be termed the "replaceable nucleotide spectrum," which is a kind of expression for the ATP binding structure. Hence, the odd fact that 2AP-TP can be replaced with ATP for both T4 RNA ligase and T4 DNA ligase supports that these enzymes have a similar AMP-binding region (on sequence analysis, T4 RNA ligase was shown to have a similar putative AMP-binding region to T4 DNA ligase), corresponding to their analogous function of ligating polynucleotides (*33*). Naturally, T4 RNA ligase as well as T4 DNA ligase could not utilize triphosphates which lack both base and sugar moieties (data not shown). Therefore, most enzymes of *moderate stringency* can be regarded to exhibit some allowance as to the sugar part but very few any as to the base part, which is different from in the case of those of *low stringency* (RNA polymerases, which exhibit allowance as to the base part but not the sugar part, are exceptional alternatives in this category of *moderate stringency*).

In summary, ATP-requiring enzymes could be classified into three categories based on the molecular parts involved in determination of the stringency: all three parts (*high stringency*), around two parts but not all (*moderate stringency*), and essentially only the phosphate part (*low stringency*). Since ATP-cognitive enzymes should recognize the triphosphate moiety due to their crucial ability of utilizing the high energy, and since they often have to discriminate, moreover, between ribo/deoxyribo, and to determine "which base" in order to complete their molecular missions, the above classification seems to be valid.

What is important is not to categorize a particular enzyme but to establish a general viewpoint that there is a strong correlation between the stringency as to ATP-requirement and the structure/function of an enzyme. This viewpoint is useful for not only tidying up our knowledge about ATP-requiring enzymes (based on the *replaceable nucleotide spectrum*), but also for providing us with an insight into the physiological and evolutionary roles of ATP.

Physiological and Evolutionary Meaning of the Replaceability—Adenosine triphosphate, besides its universal role as an energy donor, occupies the central position like a *standard material*, which should be kept constant in order to maintain the system. If ATP were altered (and subsequently dATP were also altered), the entire genetic system would face a chaotic situation resulting from aberrant Watson-Crick base pairing on replication and transcription. This effect is so fatal that almost all genes will be annihilated through unavoidable point mutations (loss of information)! Therefore, the conservation of ATP is so crucial for all life and thus ATP is definitely a *primary standard material.* Consequently, this will lead to high conservation of ATP-related proteins, especially their ATP-binding regions. The similarity between T4 RNA ligase and DNA ligases from many species (phages to man), which all carry the consensus motif, K(Y/A)DGXR (*33*), may be one demonstration of this inference. The similarity among the functionally-distant proteins of aminoacyl tRNA synthetases and firefly luciferase may be another one. In relation to this, enzymes required for ATP synthesis must be indispensible (in other words, knocking out of one of these genes is lethal). It is natural that later, today, other nucleotides (GTP, CTP, and UTP) have become equivalently essential for sustaining the complementarity of nucleic acids. In this context, the high conservation of the ATP-binding domains of AAA (ATPases Associated with a variety of cellular Activities) superfamily proteins (*34*) is quite natural. It is also reasonable, though rather simple, to think that a primordial ATP binding domain has evolved into the corresponding domains of different ATP-requiring enzymes of today, since these domains seem to be so special and indispensible.

In prokaryotic cells and the nuclei of eukaryotic cells, in which both ATP and dATP co-exist, these enzymes of *low/moderate stringency* are exposed to both nucleotides. Since the concentration of ATP (~mM) is much higher than that

of dATP, these ATP-requiring enzymes are usually fueled by ATP [The ratio of ATP/dATP is about 20 in *Escherichia coli*, and [dATP], around hundreds μM (*35*)]. However, considering the possibly similar binding constants of dATP and ATP (they exhibit a difference of a single hydrogen bond at most, while they can share tens of other interactions with cognitive enzymes), the current dATP concentration is already inhibitory. Our finding that T4 DNA ligase can utilize dATP, although less effectively than ATP, is reasonable since this property enables the enzyme to turn over a potential inhibitor for it and prevent it from being trapped with dATP. On the contrary, if the rate had been comparable with that of ATP, the consumption of dATP would not have been negligible, resulting in metabolical waste and a burden for the entire molecular system. Therefore, the present levels of stringency of most enzymes, *not too high and not too low*, must be a natural consequence. Enzymes of low stringency, such as most kinases, function in the cytosol, where there must be substantially no dATP, and they must have no necessity to discriminate ATP from dATP. In this sense, it would be challenging to investigate the local concentrations of ATP and dATP in a cell since they will reflect the nature of ATP-related enzymes. Thus, G proteins which exhibit GTPase activity need to strictly discriminate GTP from ATP since they have to function in an environment where ATP is far more abundant than GTP. Therefore, the recent X-ray observation of G protein [*H-ras* oncogene protein p21 (*36*)] that the guanine base moiety undergoes many more interactions (five bonds according to their data) with the enzyme than the adenine of ATPases [supposedly two or three in myosin (*23*)] supports our notion. Therefore, we can say that GTPase came after ATPase evolutionarily.

Technological Implications—The finding that a fluorescent molecule, 2AP-TP, can take the place of ATP is technologically important since it can be used to probe the local activities of ATP-requiring enzymes in a cell through changes in the fluorescence intensity between free and bound states. In addition, no other ATP analogous reagents are known to be fluorescent, although ones with a bulky chromophore such as a methylanthraniloyl group have been developed (*37*). Exploiting 2AP-TP (and its deoxy form), we must be able to investigate the behavior of ATP and dATP in a cell, which will lead to elucidation of the dynamic and dissipative structures in the cell, a central problem for the next stage of molecular cell biology.

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