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Characterization of nucleotide binding sites on membrane-bound chloroplast ATPase by modification with pyridoxal 5′-phosphate

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Treatment of chloroplast thylakoids with pyridoxal 5′-phosphate (PLP) in the light or in the presence of Mg^{2+} causes inhibition of photophosphorylation which is partially competitive to ADP and to inorganic phosphate (P_i), suggesting that PLP may modify essential lysine residues in the catalytic nucleotide binding site of the thylakoid H^+-ATPase. Treatment of thylakoids with PLP and NaB^3H_4 results in incorporation of about 4 mol [^{3}H]PLP/mol CF_1 almost equally distributed between α- and β-subunits. ADP plus P_i prevents incorporation of one PLP per three α-subunits and one per three β-subunits, but causes almost full protection against PLP inactivation, suggesting that PLP modification of only one α- and one β-subunit is sufficient for inactivation of the enzyme. As PLP modification of the ATPase is largely excluded in the absence of Mg^{2+}, modification of the active site may require ionic fixation of PLP with the help of the phosphate side-chain via Mg^{2+}, similar to the interaction of P_β and P_γ of ATP with the protein in order to facilitate the covalent attack to the vicinal lysine residue.

Introduction

The H^+-translocating ATPase of chloroplasts catalyzes the reversible formation of ATP from ADP and P_i. ATP formation includes cleavage of one oxygen atom from P_i, so that the bridge oxygen of the nascent β,γ-phosphate bond is contributed by the ADP molecule [1]. In 1974, Mitchell proposed a hypothesis for the molecular mechanism of ATP formation which fulfills the rules of a nucleophilic substitution (S_N2-type reaction) [2].

The stereochemistry of the ATPase reaction has been investigated with isolated F_1-ATPases of mitochondria [3] and the thermophilic bacterium PS3 [4]. The results have shown that ATP hydrolysis occurred with substituent inversion, indicating an 'in-line' phosphoryl transfer. Such transfer occurs by a pentavalent transition state, in which the binding of a hydroxyl group to γ-phosphate of ATP and cleavage of the β,γ-bond occurs simultaneously. The rule of microreversibility of enzymatic reactions requires that ATP synthesis proceeds in the same way. The enzymatic reaction needs precise orientations of the substrate molecules within the catalytic site(s) in order to facilitate the transition state involved.

Studies with chiral phosphorothioate analogues of ADP and ATP suggested that the phosphate chain of ATP or ADP, respectively, adopts a distinctly oriented stereospecific position [4,5]. The ATP molecule attains this position partly through a specific β,γ-Mg complex structure (Δ configuration). Moreover, the ADP and the ATP molecule is stereospecifically linked by a negatively charged oxygen at α-phosphorus to a positive counter-charge of the protein. This positive charge in the catalytic binding site may be contributed by the basic amino acids arginine or lysine. Several authors have concluded that essential arginines and lysines may play a role in substrate interaction with CF_1 [6–10].

This paper reports on two essential lysines, one per three α-subunits and one per three β-subunits of CF_1, which can be modified by pyridoxal 5′-phosphate in the membrane-bound enzyme. Their role in the ionic interaction with the described negative charge on P_α of ADP or ATP is discussed.

Abbreviations: P_i, inorganic phosphate; PL, pyridoxal; PLP, pyridoxal 5′-phosphate; PMS, *N*-methylphenazonium methosulfate; SDS, sodium dodecylsulfate; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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Methods

Chloroplasts were isolated from spinach leaves as described earlier [11], washed once with isolation medium and osmotically broken in a hypotonic medium containing 50 mM NaCl/2 mM Tricine buffer (pH 8.0)/1 mM $MgCl_2$. After a third wash with the same medium, the thylakoids were resuspended in 0.1 M sucrose/10 mM Tricine (pH 8.5)/5 mM $MgCl_2$/50 μM PMS, as in Ref. 8.

PLP treatment was carried out in small glass vials which were inserted into a temperature-controlled water bath on a magnetic stirrer. The temperature was 4° C and the standard light intensity (red light) 600 W/m^2. The reaction was started by addition of PLP and stopped with $NaBH_4$ after 15 min. After this procedure, the chloroplasts were washed three times in order to remove free PLP and borohydride. The washing medium contained 50 mM NaCl/25 mM Tricine (pH 8)/1 mM $MgCl_2$.

Phosphorylation was measured in a medium comprising 25 mM Tricine (pH 8)/50 mM NaCl/5 mM $MgCl_2$/50 μM PMS/5 mM ^{32}P-labeled P_i and, if not indicated otherwise, 500 μM ADP. The final volume was 0.5 ml, the temperature was adjusted to 20° C and the light intensity to 1000 W/m^2. After the illumination times indicated, the samples were stopped by addition of perchloric acid (final concn. 0.27 M) and centrifuged. [^{32}P]ATP in the supernatants was separated from P_i by precipitation of P_i by the addition of triethylamine hydrochloride and ammonium molybdate [12]. Total label and organic label were measured as Cerenkov radiation by liquid scintillation counting.

A labeled CF_1-pyridoxamine phosphate complex was obtained after PLP incubation of thylakoids and reduction of the aldimine by NaB^3H_4 (Amersham Buchler, spec. act. 185 GBq/mmol). After reduction, the thylakoids were washed three times with washing medium (see above) and resuspended in 2 M NaBr for extraction of CF_1 [13]. The subunit distribution of the label was studied in SDS-polyacrylamide gels (gradient gel, 17–22.5%) after staining with Coomassie blue. Radioactive slices containing the subunit bands of CF_1 were cut out, solved in 30% alkaline H_2O_2 [14] and measured by liquid scintillation counting.

Furthermore, membrane-bound CF_1 was labeled by [^{3}H]PLP which was prepared according to Ref. 15 by Dr. U.I. Flügge (Göttingen). The specific activity of [^{3}H]PLP was $2 \cdot 10^3$ dpm/nmol. After reduction of the aldimine by unlabeled $NaBH_4$, the protocol described above was followed.

Results

PLP is known to form a Schiff base (aldimine) with the ε-amino group of lysine. By reduction with $NaBH_4$ the relatively labile aldimine is converted into a stable secondary amine [16]. Modification of chloroplast thylakoids by 1 mM PLP in the light and in the presence of Mg^{2+} causes rapid and complete inhibition of photophosphorylation, as shown in Fig. 1. Incubation of thylakoids with PLP in the dark results in a slower and incomplete inhibition of phosphorylation.

Light via the formation of a transmembrane electrochemical proton gradient activates the ATPase which is inactive in the dark [17]. In the active state, but not in the inactive one, the catalytic site(s) of CF_1 is (are) accessible to medium nucleotides [11]. The more pronounced effect of PLP in the light may therefore indicate a rapid interaction of the label with open catalytic sites. In contrast to ADP or ATP, the smaller PLP molecule, however, seems to reach the catalytic site(s) of the inactive enzyme too, but with a lower velocity. In the dark, complete inactivation of photophosphorylation was observed only after incubation of thylakoids with high PLP concentrations (10 mM) for a long incubation time (more than 20 min) (data not shown).

Fig. 2 shows the protection of phosphorylation against PLP inactivation by the simultaneous presence of ADP, ADP plus P_i or ATP. 0.5 mM ADP is sufficient to protect thylakoids against inactivation (Fig. 2A,B). In the presence of 5 mM P_i, 0.1 mM ADP effects full protection. Compared to ADP, the protective effect of ATP (Fig. 2B) is less pronounced. The half-maximal concentrations required for protection are 160 μM for ATP and 60 μM for ADP. In the presence of 5 mM P_i, 30 μM ADP is sufficient. Phosphate alone (5 mM) shows protection of only about 30%. The lower protective effectivity of ATP coincides with the lower affinity of the enzyme for ATP in energized thylakoids [18].

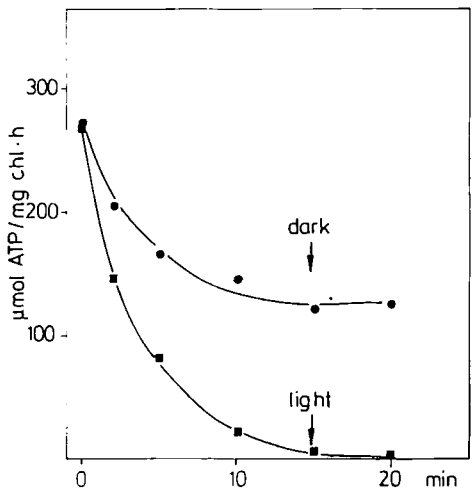

Fig. 1. Inhibition of photophosphorylation by pretreatment of chloroplasts with 1 mM PLP in the dark (●) and in the light (■). Photophosphorylation was carried out at 0.5 mM ADP, 5 mM ^{32}P-labeled phosphate, 50 μM PMS and chloroplasts equivalent to 25 μg chlorophyll/ml.

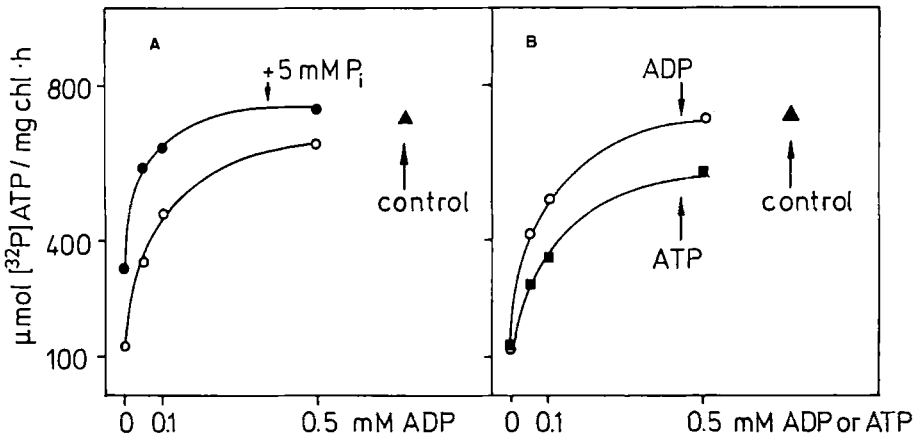

Fig. 2. Protection of photophosphorylation against PLP inactivation by adenine nucleotides and phosphate. Modification was carried out by incubation of thylakoids with 2 mM PLP in the presence of the concentrations indicated of ADP, ADP plus 5 mM P_i (A), or ATP in comparison to ADP (B) for 15 min in the light. The control (▲) indicates the activity of untreated thylakoids. Phosphorylation was measured as described in Methods, illumination time was 15 s, chloroplasts were equivalent to 25 µg chlorophyll/ml.

Addition of PLP directly into the phosphorylation assay results in partially competitive inhibition of photophosphorylation with regard to ADP (Fig. 3) and with regard to P_i (Fig. 4). This effect of PLP on photophosphorylation, together with the protection of covalent PLP modification by ADP and ATP, suggests that a part of the bound PLP molecules may cover the catalytic nucleotide binding site(s) as well as the phosphate binding site.

Treatment of thylakoids with 5 mM PLP in the light followed by treatment with NaB^3H_4 results in covalent

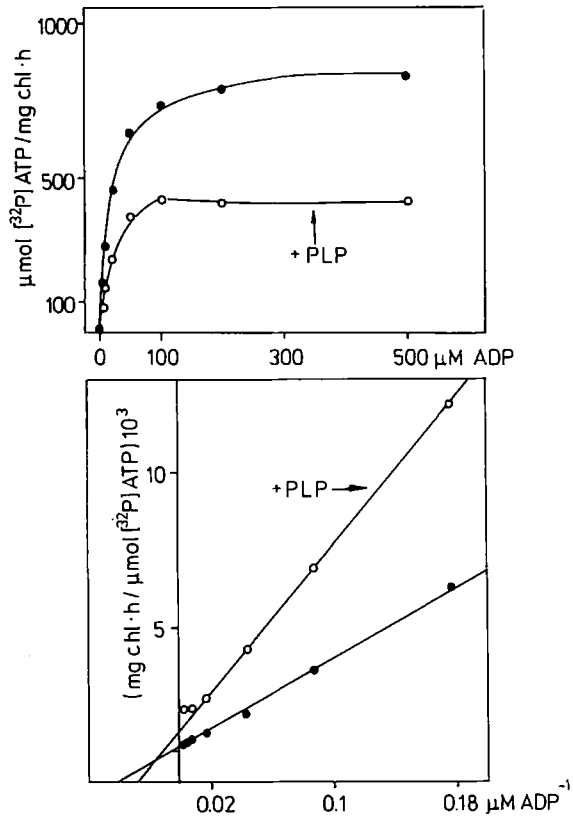

Fig. 3. Upper part: photophosphorylation as a function of ADP concentration in the absence or in the presence of 2.5 mM PLP. Phosphorylation conditions as described in Fig. 1, illumination time was 30 s. Lower part: Lineweaver-Burk plot calculated for the values shown in the upper part.

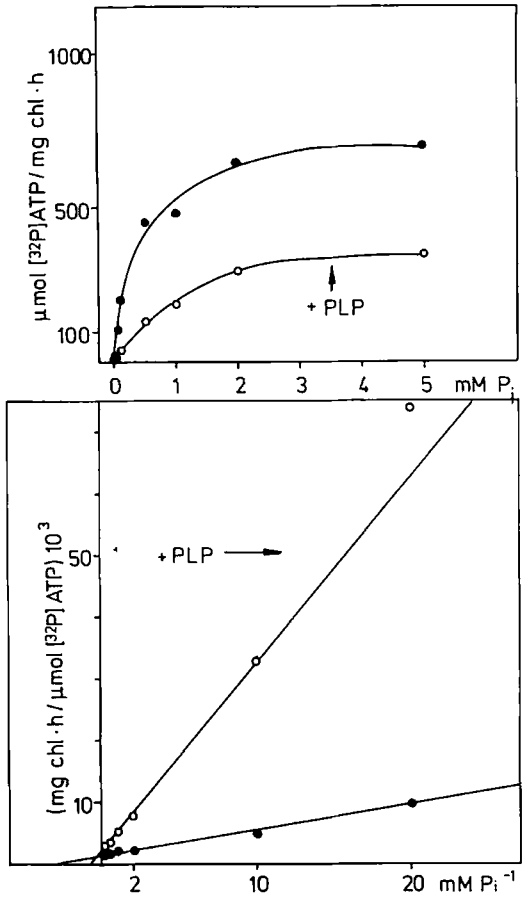

Fig. 4. Photophosphorylation as a function of P_i concentration in the absence (●) or in the presence (○) of 2.5 mM PLP. The concentration of ADP was 30 µM, other conditions as in Fig. 3.

TABLE I

Quantitative evaluation of ^3H-labeled PLP bound to separated subunits of CF_1

PLP incubation of thylakoids (15 min, red light) was followed by reduction of the aldimine by NaB^3H_4, other conditions as in Methods. A control sample without PLP was incubated with NaB^3H_4 to determine the amount of incorporated free ^3H. The values were corrected for this unspecific label.

Conditions	Mol ^3H/mol CF_1		
	total CF_1	subunits	
		α	β
5 mM PLP, light	4.2	2.4	1.8
5 mM PLP, 1 mM ADP, 10 mM P_i, light	2.8	1.5	1.3
Difference	1.5	0.9	0.5
0.5 mM PLP, light	2.4	1.4	1.0
0.5 mM PLP, 1 mM ADP, 10 mM P_i, light	0.9	0.6	0.3
Difference	1.5	0.8	0.7

incorporation of 4.2 mol [^{3}H]PLP/mol CF_1^* almost equally distributed between α- and β-subunits (Table I). Excess ADP (1 mM) plus P_i (10 mM) prevents incorporation of only about two PLP molecules per CF_1, one per three α- and one per three β-subunits (Table I), but causes almost full protection against PLP inactivation, as shown in Fig. 2.

In the presence of 0.5 mM PLP (Table I), 2.4 mol PLP/mol CF_1 are incorporated and addition of ADP and P_i again results in prevention of binding of one PLP per three α- and one PLP per three β-subunits.

Maximal PLP binding may be extrapolated to 6 mol PLP/mol CF_1, which corresponds to data reported earlier by Sugiyama and Mukohata [8]. Based on the stoichiometry of three α- and three β-subunits/CF_1, one may conclude that one nucleotide binding site per each subunit can be covalently labeled by [^{3}H]PLP. On the other hand, protection of one α- and one β-subunit against modification results in full phosphorylating activity. Provided that one PLP reacts stoichiometrically with identical lysine residues of the three subunits (β or α, respectively), we may conclude that one intact α- plus one intact β-subunit are sufficient for full activity of the H^+-ATPase. This would exclude a multiside cooperative enzyme mechanism as proposed by Boyer [19,20]. Alternatively, only one essential lysine in α- and one in β-subunits may be modified, whereas the other PLP molecules are bound to lysines which are not essential for enzyme activity.

The last possibility would be in line with the idea that three catalytic binding sites on the three β-subunits work in a strictly cooperative manner, as proposed by Boyer and Kohlbrenner [19] in their 'energy-linked binding change mechanism. These sites change their binding properties for the substrates and products in a sequential mode by conformational changes, i.e., they are in three different states at the same time. On membrane-bound CF_1 only one of these catalytic sites may be accessible to PLP, and covalent modification of this site may block the whole catalytic cycle.

In Table II the requirement of Mg^{2+} for lysine modification by PLP is shown. If chloroplasts were incubated with PLP in the light and in the presence of excess Mg^{2+} as usual, the phosphorylating activity was inhibited up to 93%, whereas omission of Mg^{2+} during modification caused only 23% inhibition. Residual inhibition in the absence of added Mg^{2+} is due to some Mg^{2+} remaining present inside the thylakoids after washing by EDTA. If EDTA (2 mM) were present in the light during the incubation procedure, no inhibition could be observed (data not shown). PLP modification in the dark and in the presence of Mg^{2+} resulted in depression of the phosphorylating activity to 64%, whereas lack of Mg^{2+} in the modification step caused only 13% inhibition.

Dark-treatment of thylakoids with non-phosphorylated PL does not inhibit photophosphorylation. Light-treatment with PL causes only 30% inhibition, irrespective of the presence or absence of Mg^{2+}. Since the Schiff-base formation between PLP and the amino group of lysine does not require Mg^{2+} [27], we conclude that lysine modification in the active site may require attachment of the phosphate residue of the modifier molecule via Mg^{2+} in order to facilitate Schiff-base formation with a vicinal lysine. This interaction is possibly similar to the interaction of the substrates P_i and β-phosphate of ADP or β- and γ-phosphate of ATP with the protein [5]. Furthermore, Mn^{2+} as well as Co^{2+} are able to

TABLE II

Photophosphorylation by thylakoids pretreated with PLP or PL in the presence or absence of Mg^{2+}

PLP or PL treatment of thylakoids was carried out with EDTA-washed membranes ± 5 mM $MgCl_2$ either in the dark or in the light. Photophosphorylation was measured in the presence of 5 mM $MgCl_2$, illumination time was 15 s, other conditions as described in Methods.

Conditions	μmol [^{32}P]ATP/mg Chl per h			
	+5 mM $MgCl_2$	% inhibition	− $MgCl_2$	% inhibition
Control, light	506.8		574.1	
+1 mM PLP, light	34.3	93	443.1	23
+1 mM PL, light	368.2	27	388.9	32
Control, dark	530.9		570.9	
+1 mM PLP, dark	191.4	64	495.2	13
+1 mM PL, dark	501.6	5	594.8	0

TABLE III

Quantitative evaluation of ^3H-labeled PLP bound to separated subunits of CF_1 in the presence or absence of Mg^{2+}

Incubation of thylakoids with [^{3}H]PLP (3.2 mM) was carried out for 15 min in red light or in the dark, for other conditions see Methods.

Conditions	mol ^3H/mol CF_1		
	total CF_1	subunits	
		α	β
Light, +10 mM $MgCl_2$	3.5	1.8	1.7
Light, without $MgCl_2$	0.8	0.4	0.4
Dark, +10 mM $MgCl_2$	2.9	1.7	1.2
Dark, Without $MgCl_2$	0.8	0.4	0.4

replace Mg^{2+} in the modification step (data not shown). These divalent cations can also replace Mg^{2+} in the process of photophosphorylation at a rate of 50% (Mn^{2+}) or 30% (Co^{2+}) of the activity in the presence of Mg^{2+} [28].

In Table III, incorporation of ^{3}H-labeled PLP into CF_1 of EDTA-washed thylakoids is compared in the absence or presence of 10 mM Mg^{2+}. Under these conditions (3.2 mM [^{3}H]PLP), 3.5 mol [^{3}H]PLP/mol CF_1 were incorporated in the presence of Mg^{2+}, whereas only 0.8 mol/mol CF_1 could be observed in the absence of Mg^{2+}. The label is almost equally distributed between α- and β-subunits in either case.

Discussion

Labeling of α-, β- and γ-subunits of CF_1 by [^{3}H]PLP was shown by Sugiyama and Mukohata [8,30] using isolated CF_1. In contrast to their results, our investigations show that labeling of γ-subunits of membrane-bound CF_1 was due to ^3H transfer by free NaB^3H_4, possibly including reduction of a disulfide bond. Labeling of γ was identical in the absence or in the presence of PLP and in the light more γ was labeled than in the dark.

In contrast to our results, which show that binding of two PLP molecules, one to one of the α- and one to one of the β-subunits, results in complete inhibition of the catalytic reaction, Sugiyama and Mukohata have reported that a stoichiometry of one PLP/CF_1 is sufficient to inactivate CF_1 [30]. The reason for this discrepancy can be found in the non-comparable experimental conditions: modification of membrane-bound CF_1 (thylakoids) and measurement of photophosphorylation (this work) versus modification of isolated CF_1 and measurement of Ca^{2+}-ATPase activity [30]. CF_1 in an isolated form is a more artificial system than membrane-bound CF_1 and it is well-known that most of the reactions of the isolated enzyme are not comparable to those of the membrane-bound form.

The nucleotide binding sites on CF_1 have been widely studied (see reviews in Refs. 21 and 22). Catalytic sites as well as 'tight' binding sites are found to be located on the β-subunits of CF_1. Some authors assume that binding sites could be formed at the interface between α- and β-subunits [23–25]. This was concluded from the labeling pattern of photoreactive nucleotide analogues like 8-azido ADP [26] and 3′-*O*-(4-benzoyl)benzoyl-ADP [24], which yielded labeling of both α- and β-subunits, particularly when membrane-bound CF_1 was the subject of investigation. The result that labeling of one lysine by PLP in the α-subunit is protected by substrates (Table I) might also support this hypothesis.

Sugiyama and Mukohata have concluded from their experiments that Mg^{2+} during light-modification of thylakoids by PLP could be required for the conformation changes in the membrane-bound CF_1 [27]. The results described here (Tables II and III) clearly show that the presence of Mg^{2+} is essential in dark-modification as well as in light-modification. For this reason, the function of the divalent cations most probably will be found in the binding mode of PLP to nucleotide binding sites and not in light-dependent conformational changes of CF_1.

In a preceding paper we have demonstrated that ATP is bound to catalytic binding sites of CF_1 as a β-, γ-Δ-Mg-ATP complex and that a negative charge on P_α of ADP and ATP interacts with a positively charged group of the protein [5]. In the catalytic site an Mg-binding carboxyl group [29] must be located, which provides interaction of P_γ and P_β of ATP. We assume

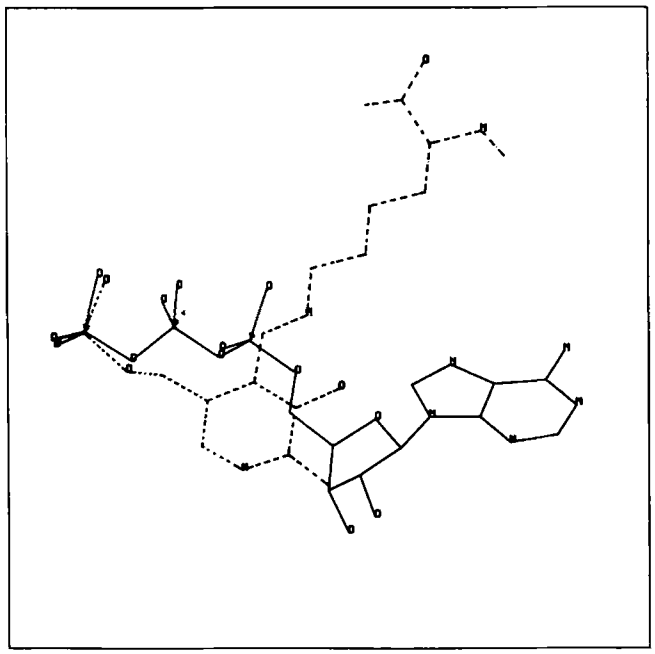

Fig. 5. Computer diagram considering correct bond angles and bond lengths of the ATP molecule (closed line) and PLP covalently bound to lysine (dotted line).

that the phosphate group of the modifier molecule PLP must be fixed by Mg^{2+} to the nucleotide binding site in a way comparable to the substrates ADP plus P_i, or ATP. In this position the reactive aldehyde group of the molecule is readable by the positive countercharge of the protein which normally interacts with the negatively charged oxygen on P_α of ATP, as can be demonstrated by comparing computer-designed model structures which include the correct bond angles and bond lengths of ATP and PLP (Fig. 5).

Tagaya et al. [31] have recently reported that two essential lysines, one in α- and one in β-subunits of *E. coli*-F_1 could be identified by bound labeled adenosine triphosphopyridoxal to be Lys-201 (α) and Lys-155 (β). The latter one is highly conserved in several nucleotide binding proteins [32] and corresponds to Lys-178 in the β-subunit of CF_1.

Whether the positive countercharge in the catalytic binding site is contributed by this lysine-178 is a matter of speculation. It has to be scrutinized by analysis of the labeling position of the pyridoxamin within the peptide sequences. These experiments are in progress.

Acknowledgements

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