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EVIDENCE FOR ESSENTIAL PRIMARY AMINO GROUPS IN A BACTERIAL COUPLING FACTOR F_1ATPASE

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SUMMARY: We have found that the binding of pyridoxal-5'-phosphate to 6 primary amino groups leads to the inactivation of the enzyme. A preferential reaction of pyridoxal-5'-phosphate with the α-subunits of this enzyme can be demonstrated. The reactivity of the amino groups is influenced by various effectors. In the presence of ATP the inhibition of the ATPase activity is noncompetitive.

INTRODUCTION: Pyridoxal-5'-phosphate (PLP) is known to form a Schiff base (an aldimine) with primary amino groups, in particular with free α-amino groups of amino acids and peptides (terminal NH_2-groups) and with the ε-amino group of lysine. By reduction with sodium borohydride the relatively labile aldimine can be converted into a stable secondary amine (1). This method allows the investigation of the binding of PLP to reactive amino groups in proteins. The first report of Fischer et al. (1) concerning the binding of PLP to muscle phosphorylase stimulated the investigation of the specific binding of PLP to a number of proteins (2-7). In 1977 Sugiyama and Mukohata found that also the membrane-bound chloroplast coupling factor 1 is inhibited by PLP (8). They later (9) reported that modification of one lysine by PLP completely inactivates the solubilized coupling factor 1 (a Ca^{2+}-ATPase). Also in 1978 Codinot et al. (10) found by using PLP as a group specific reagent that amino groups may be directly or indirectly involved in the binding of nucleotide and phosphate to the ATPase site of mitochondrial F_1ATPase. The data on the reaction of PLP with the membrane-bound and free F_1 factor from chloroplast as

PLP pyridoxal-5'-phosphate
AMP-PNP adenyl-5'-ylimidodiphosphate

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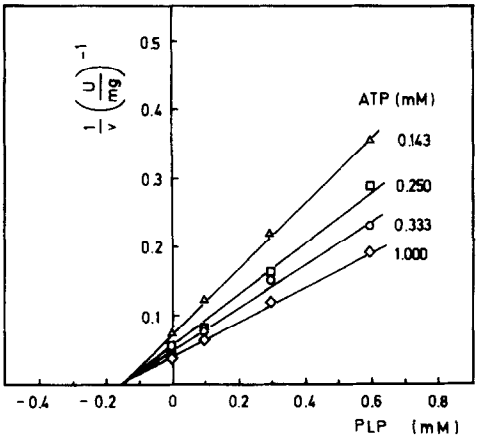

Fig. 1 Inactivation of F_1ATPase from Micrococcus species by PLP (Dixon plot).

well as with the mitochondrial F_1 factor so far permit no general conclusions concerning the function of the essential amino acid groups in these proteins.

Therefore, we have taken up some related studies on the interaction of PLP with a soluble bacterial coupling factor F_1ATPase from Micrococcus species ATCC 398.

MATERIALS AND METHODS: Isolation and preparation of F_1ATPase from Micrococcus species ATCC 398 was performed as described by Risi et al. (11). Purity and homogeneity of the enzyme were confirmed by native disc electrophoresis according to Maurer (12). The protein concentrations were determined spectrophotometrically with a Cary spectrophotometer (type 118c) using an absorption coefficient of $A^{1\%}_{280} = 6.0$ (11) and assuming a molecular weight of 380 000 D ± 50 000 D (13). The ATPase activity was assayed by measuring the release of inorganic phosphate after the method of Fiske and Subbarow (14) modified by Arnold et al. (15). For the determination of the inhibition type, 1 µg F_1ATPase was incubated at 37 °C in 5 ml 4-morpholinopropane sulfonic acid-buffer (50 mM, pH 7.5) with different PLP concentrations. The reaction was started by adding Ca^{2+} and ATP (molar ratio 5:1). ^{3}H-Labeled PLP (specific activity 56.6 Ci/mol) was prepared after the method of Stock et al. (16).

The reactivation experiments were conducted 1.) by diluting 1:1000 an aliquot of an incubation mixture of F_1ATPase (0.1 mg/ml) and PLP (5×10^{-4} M) or 2.) by adding lysine (10^{-2} M) to the incubation mixture mentioned above.

Sodium dodecyl sulfate gel electrophoresis was carried out as described earlier (17).

RESULTS: Figure 1 shows that PLP inhibits the F_1ATPase from Micrococcus species in a noncompetitive manner. An inhibitor constant $K_i = 1.4 \pm 0.1 \times 10^{-4}$ M is deduced from the kinetic measurements. The formation of the enzyme inhi-

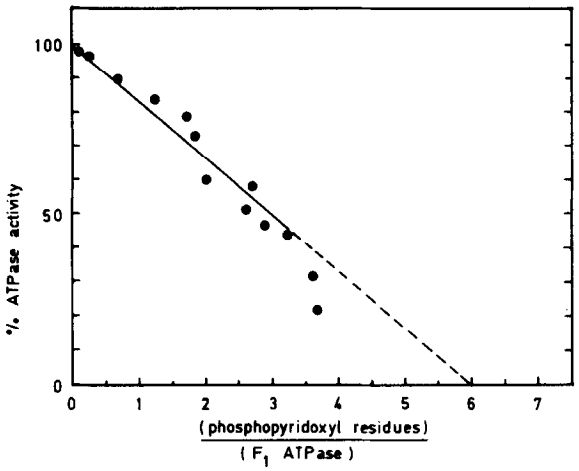

Fig. 2 Inactivation of F_1ATPase from Micrococcus species as a function of the number of PLP residues bound per enzyme molecule. 0.15 mg aliquots of F_1ATPase were incubated at 37 °C in the presence of PLP for 15 min as described in "MATERIALS AND METHODS". Then a 1 M solution of $NaBH_4$ (in the same buffer) was added (25 fold excess over PLP) at 0 °C. Reduction was achieved by incubation for 15 min at 0 °C and then for 30 min at 20 °C. Activity determinations were performed after transferring aliquots to the activity assay system (dilution 1 : 1000). The modified enzyme was extensively dialyzed for 24 h against several changes of the standard buffer containing 0.2 M NaCl. The incorporation of PLP was calculated from the absorption spectrum of the modified enzyme.

bitor complex can be reversed by dilution (1:1000) or by addition of 10^{-2} M lysine. Pyridoxamine exhibits no inhibitory effects at concentrations up to 10^{-2} M. The difference spectrum of the F_1 factor/PLP complex (measured against equimolar PLP) equals that of the corresponding poly-L-lysine/PLP complex. By sodium borohydride reduction of the F_1 factor/PLP complex the absorption peak at 430 nm vanishes and a new, though weaker, absorption band at 325 nm appears. By using a molar extinction coefficient of 1×10^4 $M^{-1}cm^{-1}$ at 325 nm (18) the number of PLP bound per F_1ATPase could be calculated. Figure 2 shows the inactivation of the F_1ATPase as a function of the number of PLP residues bound per enzyme molecule. A linear correlation is evident above a residual activity of 40%. Extrapolation of the linear part yields an intersect at 6 PLP per F_1 factor. Under the present experimental conditions, however, only up to 3.7 ± 0.2 PLP residues could be incorporated per F_1ATPase.

TABLE 1: Influence of different effectors on the incorporation of $|^3H|$PLP in the α- and β-subunits of F_1ATPase from Micrococcus species

Effector	Ratio of incorporation α:β
-	6.1
Mg^{2+} (1 mM)	6.0
PO_4^{3-} (1 mM)	7.6
AMP-PNP (0.01 mM), Mg^{2+} (0.01 mM)	6.2
ATP (1 mM), Mg^{2+} (1 mM)	8.5
ADP (1 mM), Mg^{2+} (1 mM)	10.5

In corresponding experiments $|^3H|$PLP was incorporated into the enzyme and subjected to sodium borohydride reduction. When the reduced $|^3H|$PLP/enzyme complex was subjected to sodium dodecyl sulfate gel electrophoresis (19) it could be found that most $|^3H|$-activity migrated with the α-subunit. After incorporation of about 3 PLP per enzyme molecule (40% residual activity) in the absence of effectors the α-subunit was labeled about 6 fold in comparison to the β-subunit. Table 1 shows the influence of various effectors on the labeling of the α- and β-subunits of the bacterial F_1ATPase. All effectors caused a more specific labeling of the α-subunit. Most pronounced were the effects produced by MgATP. The absolute rate of PLP incorporation was only slightly changed by the various effectors.

DISCUSSION: The noncompetitive inhibition type indicates that PLP is not directly bound to substrate binding sites or catalytic centers. The reversibility of the inhibition is compatible with the conclusion that PLP primarily yields an aldamine or a Schiff base (aldimine) by reaction with primary amino groups, very probably ε-amino groups of lysine. An additional stabilisation of the PLP-enzyme compound with the help of the phosphate side chain appears to be of minor importance. The inhibitory strength of pyridoxal is 1/25 that of pyridoxal-5'-phosphate. Possibly, this lower inhibitory effect of pyridoxal is largely due to its prevailing semiacetal structure that reduces the reactivity of the aldehyde group (7). ATP binding sites at the β-subunit of the

present F_1 ATPase have been identified with various substrate analogues (13, 19). Recent data indicate that the catalytic center of this F_1ATPase is probably located at interfaces between α- and β-subunits (20). The reactive amino groups at the α-subunits could be part of an anion binding site, perhaps a phosphate binding site. According to Slater et al. (21) also the mitochondrial F_1ATPase has two anion binding sites at the α-subunit.

For the chloroplast F_1 coupling factor the incorporation of PLP is much less specific. Labeling of α-, β- and γ-subunits has been reported (8, 9).

It is expected that the membrane-bound F_1ATPase will respond to PLP in a different way. This has been shown for the chloroplast coupling factor 1 (8, 9). Such comparative data may give more clues to the function of the essential amino groups.

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