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Photoaffinity cross-linking of F_1ATPase from the thermophilic bacterium PS3 by 3′-arylazido-β-alanyl-2-azido ATP

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The photoactivatable bifunctional 3′-arylazido-β-alanyl-2-azido ATP (2,3′-DiN_3ATP) has been applied to study the localization of the nucleotide-binding sites of coupling factor 1 (F_1ATPase, TF_1) from the thermophilic bacterium PS3 by photoaffinity cross-linking. UV irradiation of TF_1 in the presence of 2,3′-DiN_3ATP results in the nucleotide-dependent formation of various higher molecular mass cross-links formed by two, three or even four α- and/or β-subunits. The differences observed upon photoaffinity cross-linking by the bifunctional 2-azido ATP or 8-azido ATP analog are discussed. They are probably due to the varied maximal distance between both azido groups, or to the different conformations (*anti*/*syn*) of these analogs. The results confirm our suggestion that several (possibly all) nucleotide-binding sites of F_1ATPases are located at the interfaces between α- and β-subunits.

ATPase, F_1-; Nucleotide conformation; Photoaffinity crosslinking; Interfacial localization; Nucleotide-binding site

1. INTRODUCTION

Strong catalytic site cooperativity has been demonstrated for the synthesis/hydrolysis of ATP catalyzed by ATP synthase complexes [1–5]. The catalytic part (F_1ATPase) of this enzyme from most species has a subunit composition of $\alpha_3\beta_3\gamma\delta\epsilon$ with up to six, probably three catalytic and three noncatalytic, nucleotide-binding sites on the major subunits α and/or β. These data have been substantiated by binding studies, affinity labeling and photoaffinity labeling [6–8]. For an effective cooperativity between catalytic/noncatalytic nucleotide-binding sites, subunit-subunit interactions are essential. The localization of these binding sites at interfaces between α- and β-subunits yields an attractive model which implies strong subunit-subunit interactions. Such a model has been proposed and discussed by several authors [9–16]. All catalytic or regulatory events at interfacial sites should directly influence the adjacent subunits. First experimental evidence for an interfacial localization has been obtained by photoaffinity cross-linking of various ATP synthase complexes with the bifunctional photoactivatable 8,3′-DiN_3ATP [17–20]. The irradiation of F_1- or F_0F_1ATPases in the presence of 8,3′-DiN_3ATP resulted in the nucleotide-specific formation of α-β cross-links. Furthermore, the formation of even higher molecular mass cross-links composed by three or probably four α- and β-subunits could be observed, indicating that more than one nucleotide-binding site is located at interfacial domains of α- and β-subunits [19]. The preferential *syn*-conformation of 8-azido ATP derivatives may be disadvantageous for an efficient binding of 8,3′-DiN_3ATP to F_1ATPases

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Abbreviations: TF_1, coupling factor 1 (F_1ATPase) from the thermophilic bacterium PS3; 2,3′-DiN_3ATP, 3′-arylazido-2-azido ATP, 3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl} 2-azidoadenosine 5′-triphosphate; 8,3′-DiN_3ATP, 3′-arylazido-8-azido ATP, 3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl} 8-azidoadenosine 5′-triphosphate

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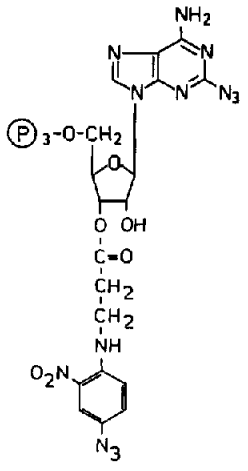

Fig.1. Structural formula of 2,3′-DiN$_3$ATP.

[8,21]. Especially at the noncatalytic nucleotide-binding sites which are highly specific for ATP and ADP, more specific than the catalytic sites, a considerable labeling by 8-azidoadenine nucleotides has not been observed so far. To exclude this disadvantage we have synthesized 2,3′-DiN$_3$ATP (fig.1) [22]. This photoreactive bifunctional ATP analog should be preferentially in the *anti*-conformation like ATP [23]. Here we report on photoaffinity cross-linking of the F$_1$ATPase (TF$_1$) from the thermophilic bacterium PS3 with 2,3′-DiN$_3$ATP. TF$_1$ is more stable and does not contain tightly bound nucleotides in comparison with F$_1$ATPases from mesophilic species [24]. This property is expected to ease the accessibility of the photoaffinity label to all catalytic and noncatalytic nucleotide-binding sites.

2. MATERIALS AND METHODS

2.1. *Preparation of F$_1$ATPase (TF$_1$) from the thermophilic bacterium PS3*

TF$_1$ was prepared from plasma membranes of PS3 as described earlier [25]. The absence of tightly bound nucleotides was tested by HPLC after acid denaturation, UV absorption, phosphate analysis, or ^{31}P-NMR spectroscopy. ATPase activity was determined by continuous measurement of the liberated phosphate at 60°C in 5 ml test solution containing 0.5 μg TF$_1$, 100 mM Tris-HCl (pH 8.0), 5 mM Ca^{2+} and 1 mM ATP [26]. The protein concentration was measured according to Lowry et al. [27].

2.2. *Photoaffinity cross-linking*

2,3′-DiN$_3$ATP was synthesized by esterification of *N*-4-azido-2-nitrophenyl-β-alanine with 2-azido ATP as described earlier [22] according to [17,28]. Photoaffinity cross-linking was performed by irradiation of TF$_1$ (usually 100 μg) in 500–1000 μl Tris-HCl buffer (100 mM, pH 8.0) with a Zeiss LX 501 spectrophotometer (λ = 310 nm), or with a Mineralight handlamp UVSL 25 (long wavelength; maximal emission at 360 nm) in the presence of 0.02–0.05 mM·2,3′-DiN$_3$ATP at 37°C. The separation of the cross-linked proteins by SDS-gel electrophoresis and the determination of the cross-link composition by hydrolytic cleavage was performed as described earlier [18,19].

3. RESULTS AND DISCUSSION

3.1. *Specific interaction of 2,3′-DiN$_3$ATP with TF$_1$*

The specific interaction of a photoaffinity label with an enzyme is ideally demonstrated if the photoreactive analog is a substrate or at least a competitive inhibitor in the dark. TF$_1$ hydrolyzed 2,3′-DiN$_3$ATP in the presence of Mg^{2+}. The rate of hydrolysis of Mg·2,3′-DiN$_3$ATP (1.5 μmol P$_i$/min per mg protein) was about 5% of the hydrolysis rate of Mg·ATP. Hydrolysis of 2,3′-DiN$_3$ATP could not be observed in the presence of Ca^{2+}. These results agree with those obtained for the hydrolytic cleavage of 8,3′-DiN$_3$ATP [18,19] and several other 2′- or 3′-substituted ATP analogs [3,4,29] by various F$_1$ATPases.

The specific interaction of 2,3′-DiN$_3$ATP could also be confirmed by its competitive inhibition of ATP hydrolysis at higher ATP concentrations ([ATP] > 2.5 × 10^{-4} M) (fig.2). At lower ATP concentrations the Lineweaver-Burk plot does not indicate a competitive inhibition. This effect was not observed for 8,3′-DiN$_3$ATP which inhibits TF$_1$ competitively at all ATP concentrations tested [19]. F$_1$ATPase from *Micrococcus luteus* shows an analogous behavior for the hydrolysis of ATP in the presence of 2,3′-DiN$_3$ATP [22]. These results could be caused by different conformations (*anti/syn*) of the nucleotides: 2,3′-DiN$_3$ATP may also interact with noncatalytic nucleotide-binding sites whereas the 8-azido analog binds efficiently only to the catalytic sites.

3.2. *Light-induced inactivation of TF$_1$ by 2,3′-DiN$_3$ATP*

The ATPase activity of TF$_1$ was drastically inhibited by UV irradiation in the presence of 2,3′-DiN$_3$ATP (fig.3). In comparison with

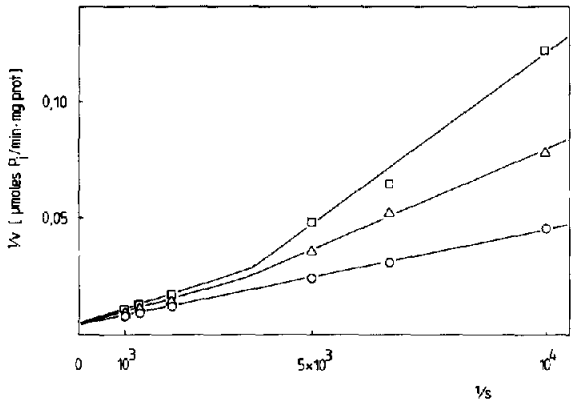

Fig.2. The effect of 2,3′-DiN$_3$ATP on the hydrolysis of ATP. Plots of $1/v$ vs $1/[Ca \cdot ATP]$ of TF_1 in the absence of 2,3′-DiN$_3$ATP (○) and in the presence of 2,3′-DiN$_3$ATP [5 µM (△); 10 µM (□)]. ATPase activity was determined at 60°C in 5 ml test solution containing 0.5 µg TF_1, 100 mM Tris-HCl (pH 8.0), different concentrations of 2,3′-DiN$_3$ATP and Ca·ATP ($[Ca^{2+}]/[ATP] = 5:1$).

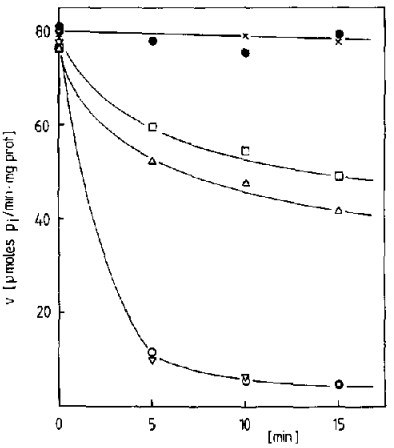

Fig.3. Light-induced inhibition of TF_1. Irradiation in the presence of 0.05 mM Mg·2,3′-DiN$_3$ATP (○), dark control in the presence of 0.05 mM Mg·2,3′-DiN$_3$ATP (●), light control in the absence of 2,3′-DiN$_3$ATP (×); irradiation in the presence of 0.05 mM Mg·2,3′-DiN$_3$ATP and 1 mM Mg·ATP (□), 1 mM Mg·ADP (△), or 1 mM Mg·AMP (▽), respectively. ATPase activity was determined at 60°C in 5 ml test solution containing 0.5 µg TF_1, 100 mM Tris-HCl (pH 8.0), 5 mM Ca^{2+} and 1 mM ATP.

8,3′-DiN$_3$ATP this inactivation already occurs at substantially lower concentrations of the photoaffinity label indicating that the interactions of 2,3′-DiN$_3$ATP with TF_1 are more effective than those of the 8-azido analog. This effect agrees with the favorable *anti*-conformation of 2,3′-DiN$_3$ATP. The enzyme remained active upon dark incubation in the presence of 2,3′-DiN$_3$ATP (dark control) as well as upon UV irradiation in the absence of the label (light control). The light-induced inactivation of TF_1 by 2,3′-DiN$_3$ATP could be prevented partially by the prior addition of ATP or ADP. Both compete with 2,3′-DiN$_3$ATP for the nucleotide-binding sites. AMP, which is not bound specifically to TF_1 did not influence the photoinactivation. The protection of the enzyme by ADP or ATP indicates the nucleotide specificity of the labeling by 2,3′-DiN$_3$ATP.

3.3. *Photoaffinity cross-linking of TF_1 by 2,3′-DiN$_3$ATP*

The UV-induced inactivation of TF_1 in the presence of 2,3′-DiN$_3$ATP also resulted in the formation of two-subunit cross-links (m > 100 kDa) (fig.4; fig.6, gel b, region 3). Addition of ATP or ADP prior to the photoactivation of the label decreased the formation of these cross-links whereas addition of AMP did not show any effect (fig.5). These data agree with the results observed for the photoinactivation of TF_1 by 2,3′-DiN$_3$ATP (fig.3) and demonstrate the nucleotide specificity of the cross-link formation, too. The electrophoretic mobility of the two-subunit cross-links and of their hydrolytic cleavage products indicate that these cross-links are composed by α- and/or

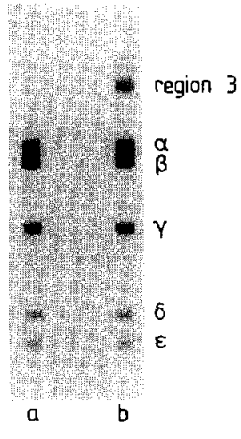

Fig.4. Photoaffinity cross-linking of TF_1. SDS electrophoresis gels of labeled (cross-linked) TF_1 (50 µg): a, native TF_1 (control); b, TF_1 labeled by 0.05 mM Mg·2,3′-DiN$_3$ATP.

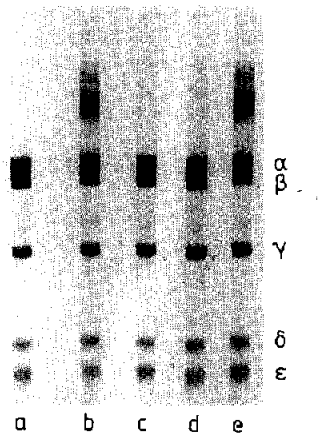

Fig.5. The influence of added effectors on the formation of cross-links. SDS electrophoresis gels of TF_1 (50 μg) labeled by 0.05 mM 2,3′-DiN_3ATP in the presence of: b, 0.05 mM Mg^{2+}; c, 1.05 mM Mg^{2+} and 1 mM ATP; d, 1.05 mM Mg^{2+} and 1 mM ADP; e, 1.05 mM Mg^{2+} and 1 mM AMP; a, native TF_1 (control).

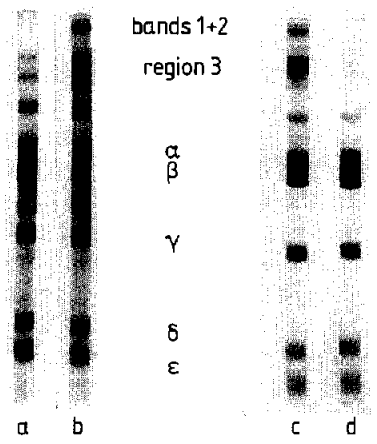

Fig.6. Photoaffinity cross-linking of TF_1 by 2,3′-DiN_3ATP and 8,3′-DiN_3ATP, respectively. SDS electrophoresis gels of TF_1: a, native TF_1 (200 μg) (control); b, TF_1 (200 μg) labeled by 0.5 mM Mg·2,3′-DiN_3ATP; c, TF_1 (100 μg) labeled by 0.5 mM Mg·8,3′-DiN_3ATP; d, native TF_1 (100 μg) (control).

β-subunits. Besides the two-subunit cross-links even higher molecular mass protein bands (three-subunit cross-links or probably four-subunit cross-links) became visible when higher amounts of the labeled protein were applied onto one gel (fig.6, gel b, bands 1 + 2).

The comparison of the gel patterns obtained by SDS electrophoresis of TF_1 cross-linked by either 2,3′-DiN_3ATP or 8,3′-DiN_3ATP shows a great conformity. The bands 1 and 2 and the region 3 can be observed in both cases (fig.6, gels b + c). The decrease of the yields of these cross-links with the increasing number of cross-linked subunits is conclusive. For the cross-linking of two subunits, two azido groups of one label have to be in a proper position to bind covalently to amino acid residues of two adjacent subunits immediately upon photoactivation. Four azido groups of two labels must be well positioned to cross-link three subunits. The chance for cross-linking four subunits by three labels is very low. Six azido groups have to be involved in the formation of such a cross-link.

When applying smaller amounts of the labeled protein onto an electrophoresis gel, however, the heterogeneity of region 3 became evident due to the formation of various two-subunit cross-links. Yet, the yield of these cross-links differs remarkably for the two analogs (fig.7). Upon photoaffinity cross-linking of TF_1 by 8,3′-DiN_3ATP the upper band is more intensive than the second band whereas it is vice versa for 2,3′-DiN_3ATP. This difference can be explained easily. Firstly, both azido groups of 2,3′-DiN_3ATP may be about 0.2 nm more apart than those of the 8-azido analog. Secondly, 2,3′-DiN_3ATP should involve the noncatalytic as well as the catalytic nucleotide-binding sites of TF_1. 8,3′-DiN_3ATP, however, is expected to bind

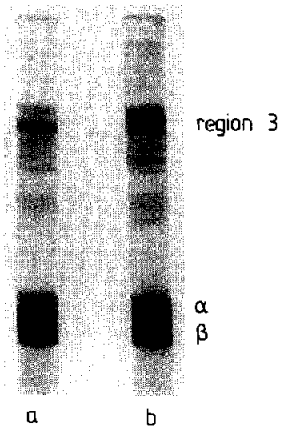

Fig.7. Differences in the formation of two-subunit cross-links (region 3) obtained by irradiation of TF_1 (70 μg) in the presence of 0.5 mM Mg·2,3′-DiN_3ATP (a), or 0.5 mM Mg·8,3′-DiN_3ATP (b).

efficiently only to the catalytic sites as discussed above. Both facts should cause the labeling of different amino acid residues at the catalytic and/or noncatalytic nucleotide-binding sites by 2,3′-Di N_3ATP and 8,3′-DiN_3ATP resulting in a different composition and a different electrophoretic mobility of the two-subunit cross-links. This has been demonstrated for photoaffinity labeling of various F_1ATPases from mitochondria, bacteria and chloroplasts by the monofunctional 2- and 8-azidoadenine nucleotides [30–35].

Our results demonstrate the suitability of both bifunctional diazido ATP analogs. The described differences between photoaffinity cross-linking by 2,3′-DiN_3ATP and 8,3′-DiN_3ATP are advantageous for a further differentiation between catalytic and noncatalytic nucleotide-binding sites of ATP synthases.

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REFERENCES

[1] Hutton, R.L. and Boyer, P.D. (1979) J. Biol. Chem. 254, 9990–9993.
[2] Gresser, M.J., Myers, J.A. and Boyer, P.D. (1982) J. Biol. Chem. 257, 12030–12038.
[3] Grubmeyer, C. and Penefsky, H.S. (1981) J. Biol. Chem. 256, 3718–3727.
[4] Grubmeyer, C. and Penefsky, H.S. (1981) J. Biol. Chem. 256, 3728–3734.
[5] Cross, R.L. (1981) Annu. Rev. Biochem. 50, 681–714.
[6] Cross, R.L. and Nalin, C.M. (1982) J. Biol. Chem. 257, 2874–2881.
[7] Cross, R.L. (1988) J. Bioenerg. Biomembr. 20, 395–405.
[8] Vignais, P.V. and Lunardi, J. (1985) Annu. Rev. Biochem. 54, 977–1014.
[9] Klein, G., Lunardi, J., Satre, M., Lauquin, G.J.M. and Vignais, P.V. (1977) in: Structure and Function of Energy-Transducing Membranes (Van Dam, K. and Van Gelder, B.F. eds) pp.283–293, Elsevier/North-Holland Biomedical Press, New York.
[10] Kozlov, I.A. and Skulachev, V.P. (1977) Biochim. Biophys. Acta 463, 29–89.
[11] Bragg, P.D., Stan-Lotter, H. and Hou, C. (1981) Arch. Biochem. Biophys. 207, 290–299.
[12] Bruist, M.F. and Hammes, G.G. (1981) Biochemistry 20, 6298–6305.
[13] Williams, N. and Coleman, P.S. (1982) J. Biol. Chem. 257, 2834–2841.
[14] Senior, A.E. and Wise, J.G. (1983) J. Membr. Biol. 73, 105–124.
[15] Gromet-Elhanan, Z. and Khananshvili, D. (1984) Biochemistry 23, 1022–1028.
[16] Kambouris, N.G. and Hammes, G.G. (1985) Proc. Natl. Acad. Sci. USA 82, 1950–1953.
[17] Schäfer, H.-J., Scheurich, P., Rathgeber, G., Dose, K., Mayer, A. and Klingenberg, M. (1980) Biochem. Biophys. Res. Commun. 95, 562–568.
[18] Schäfer, H.-J. and Dose, K. (1984) J. Biol. Chem. 259, 15301–15306.
[19] Schäfer, H.-J., Rathgeber, G., Dose, K., Masafumi, Y. and Kagawa, Y. (1985) FEBS Lett. 186, 275–280.
[20] Schäfer, H.-J., Mainka, L., Rathgeber, G. and Zimmer, G. (1983) Biochem. Biophys. Res. Commun. 111, 732–739.
[21] Sarma, R.H., Lee, C.H., Evans, F.E., Yathindra, H. and Sundaralingam, M. (1974) J. Am. Chem. Soc. 96, 7337–7348.
[22] Schäfer, H.-J., Rathgeber, G., Dose, K., Sauer, H.E. and Trommer, W.E. (1989) Z. Naturforsch., submitted.
[23] Czarnecki, J.J. (1984) Biochim. Biophys. Acta 800, 41–51.
[24] Yoshida, M. and Allison, W.S. (1983) J. Biol. Chem. 258, 14407–14412.
[25] Kagawa, Y. and Yoshida, M. (1979) Methods Enzymol. 55, 781–787.
[26] Arnold, A., Wolf, H.U., Ackermann, B. and Bader, H. (1976) Anal. Biochem. 71, 209–213.
[27] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[28] Jeng, S.J. and Guillory, R.J. (1975) J. Supramol. Struct. 3, 448–468.
[29] Schäfer, G., Onur, G. and Schlegel, M. (1980) J. Bioenerg. Biomembr. 12, 213–233.
[30] Hollemanns, M., Runswick, M.J., Fearnly, I.M. and Walker, J.E. (1983) J. Biol. Chem. 258, 9307–9313.
[31] Garin, J., Boulay, F., Issartel, J.P., Lunardi, J. and Vignais, P.V. (1986) Biochemistry 25, 4431–4437.
[32] Cross, R.L., Cunningham, D., Miller, C.G., Xue, Z., Zhou, J.-M. and Boyer, P.D. (1987) Proc. Natl. Acad. Sci. USA 84, 5715–5719.
[33] Xue, Z., Zhou, J.-M., Melese, T., Cross, R.L. and Boyer, P.D. (1987) Biochemistry 26, 3749–3753.
[34] Xue, Z., Miller, C.G., Zhou, J.-M. and Boyer, P.D. (1987) FEBS Lett. 223, 391–394.
[35] Wise, J.G., Hicke, B.J. and Boyer, P.D. (1987) FEBS Lett. 223, 395–401.