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Photoaffinity labeling and photoaffinity crosslinking of enzymes

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Photoaffinity labeling is a special type of chemical modification, where the label is activated by the action of light. This article presents the general principles and limitations of this technique, its application to the study of ***Micrococcus luteus*** *ATPase and the use of photoaffinity crosslinking to probe the structure of this enzyme.*

Key terms: *F_1ATPase; photoaffinity crosslinking; photoaffinity labeling;* ***Micrococcus luteus.***

INTRODUCTION

This article is divided into two main parts. The first part, presenting the general principles of photoaffinity labeling, is based mainly on review articles (Knowles, 1972; Bayley & Knowles, 1977) and on a chapter of an excellent laboratory manual (Bayley, 1983). The second part demonstrates the application of photoaffinity labeling and photoaffinity crosslinking with a special enzyme, as performed in our laboratory.

PHOTOAFFINITY LABELING

Advantages of photoaffinity labeling

Chemical modification is often applied to the study of interactions of receptor molecules with their ligands in biological systems.

$$\text{receptor} + \text{ligand} \rightarrow [\text{receptor} \bullet \text{ligand}]$$

The receptor binds its ligand usually non-covalently at a specific binding site to form a receptor • ligand complex. Receptors are generally proteins or protein conjugates (*e.g.*, glycoproteins) like enzymes, immunoglobulins and receptors for hormones, neurotransmitters, or drugs. The corresponding ligands are enzyme substrates, cofactors, allosteric effectors, antigens, hormones, neurotransmitters, or drugs. In contrast to the receptors, these ligands differ widely in their molecular structure. Among them are all products of cellular metabolism like sugars, amino acids, nucleotides, and oligomers of these compounds and synthetic products like drugs.

The elucidation of the ligand binding site often allows conclusions about the structure and the function of the receptor protein. One approach to the characterization of a ligand binding site is chemical modification. The first successful results in modifying receptor proteins have been obtained by application of group-specific reagents [Fig 1] (Means & Feeney, 1971; Glazer *et al*, 1975). These substances react specifically with definite amino acid residues of the protein, ideally only with one distinct residue. Group-specific reagents normally attack only nucleophilic groups of the receptor protein. In addition to this, group-specific reagents often do not discriminate between amino acid residues inside or outside of the ligand binding site. The inactivation of the receptor's function is only a weak indication of the modification of an essential amino acid residue inside the ligand binding site. The inhibition could also be caused by a conformational change of the

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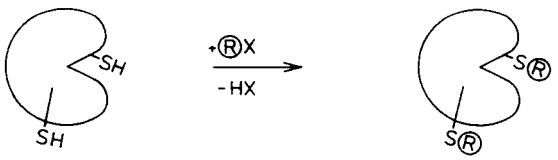
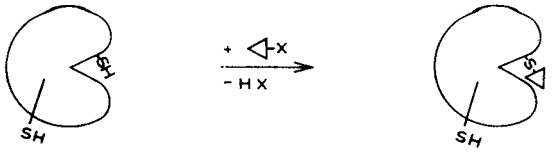


active inactive

Fig 1. Modification of proteins by group-specific reagents (X).



active inactive

Fig 2. Affinity labeling of receptor proteins (Δ-X: affinity label).

protein due to a modification distant from the ligand binding site.

The specific labeling of the binding site is favored by a local increase in the modifying reagent at the ligand binding site. This can be achieved by the incorporation of the reactive group-specific reagent into a ligand creating a reactive ligand analog (affinity label). This analog possesses a certain affinity to the receptor protein allowing a specific interaction at the ligand binding site. There it can bind covalently to a nucleophilic amino acid residue due to its reactive group (affinity labeling) [Fig 2] (Jacoby & Wilchek, 1977). Unfortunately, affinity labels, analogous to group-specific reagents, react exclusively with nucleophilic amino acids. However, there are often hydrophobic amino acids involved in the binding of ligands which are not labeled by group-specific reagents or affinity labels with conventional functional groups. Furthermore, affinity labels react immediately after their addition to the system inactivating the receptor owing to the formation of covalent bonds. For this reason it is difficult to study the specific biological interactions of these analogs. Owing to the electrophilic character of their reactive groups most of the added affinity labels are very often hydrolyzed by the solvent water before reaching their target.

These disadvantages are eliminated when using a non-reactive precursor which can be activated at will. These reagents do not bind covalently to the protein unless activated. Usually photoactivable ligand analogs are applied for this purpose (photoaffinity labeling) [Fig 3] (Knowles, 1972; Bayley & Knowles, 1977; Bayley, 1983). Since they are chemically inert in the dark, it is easy to study their biological interactions under these conditions. Upon irradiation of these precursors, highly reactive intermediates are formed which react indiscriminately with all surrounding groups. After its activation, a photoaffinity label interacting at the specific binding site is capable of labeling all amino acid residues of the binding area.

The advantages of photoaffinity labeling are:

a) Photoaffinity labels are chemically inert in the dark.
b) The time and the rate of the labeling can easily be controlled by irradiation.
c) The photogenerated intermediates are highly reactive, resulting in a labeling of hydrophobic as well as nucleophilic amino acid residues. All amino acids of proteins can thus be labeled.

Criteria for photoaffinity labels

A photoactivable reagent has to fulfill the following criteria to be an ideal photoaffinity label:

a) The photoaffinity label should be chemically inert in the dark. All conditions of the labeling experiment, like temperature, pH value and the redox potential of the chemicals present in the mixture, should not damage the photoactivable precursor.
b) It should easily be synthesized with good yields. This criterion is especially important for the synthesis of radiolabeled ligand analogs.
c) The chemical changes necessary to make a ligand photoreactive should not alter its conformation too drastically. Modification by bulky photoactivable compounds may decrease the affinity of the ligand to the receptor protein to an extend where there are no specific biological interactions to allow a specific labeling.

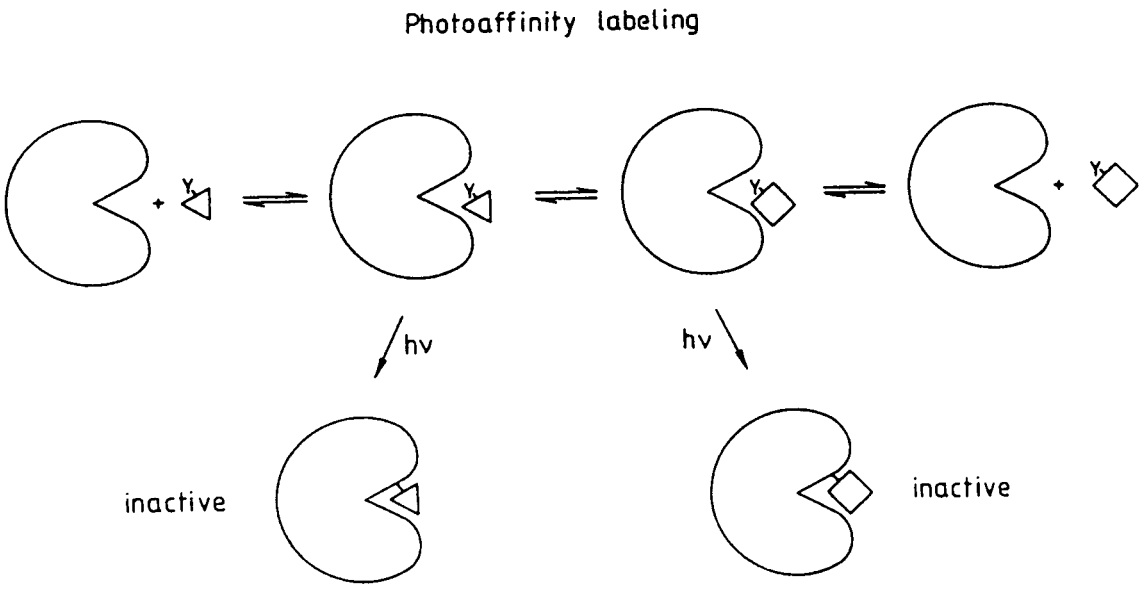


Fig 3. Photoaffinity labeling of enzymes (Δ-Y: substrate analog; suare-Y: product analog).

d) The photoaffinity label should be activated by light of a wavelength which does not damage the other components of the biological system like proteins or nucleic acids. Therefore, it is desirable to perform the irradiation with light of wavelengths longer than 300 nm.
e) The photogenerated intermediate should be highly reactive. Its lifetime has to be very short to guarantee immediate labeling at the place of its formation. For this reason, all reactive intermediates that rearrange to less reactive species should be avoided.
f) The highly reactive intermediate should form stable bonds with the receptor protein allowing all the analytical procedures required for the characterization of the labeled protein.

It should be noted that there is nearly no photoaffinity label which completely fulfills each of these criteria.

Photogenerated reactive intermediates

Two types of reactive intermediates can be generated by irradiation of relatively inert precursors. The first group is produced by the homolytic cleavage of a single bond resulting in two free radicals or one diradical. The second group involves carbenes and nitrenes. They are generated upon homolytic cleavage of a double bond or two adjacent single bonds. Carbenes contain a divalent carbon and nitrenes a monovalent nitrogen [Fig 4]. These intermediates are highly reactive because of their lack of electrons. Free carbon radicals possess only seven, nitrenes or carbenes only six electrons in their outer electron shell. All try vehemently to complete an electron octet.

Carbenes. A carbene formed by irradiation of diazoacetyl chymotrypsin was the first reactive intermediate applied to photoaffinity labeling (Singh *et al*, 1962). Generally, carbenes can react with polar residues as well as with hydrophobic residues. For this reason carbenes can be applied not only to labeling active sites of enzymes, which usually contain nucleophilic amino acid residues, but also to hydrophobic regions of both receptor binding sites and membrane-integrated proteins. Carbenes react with nucleophilic groups of alcohols or amines yielding, for example, an ether or a secondary amine. Furthermore, they either

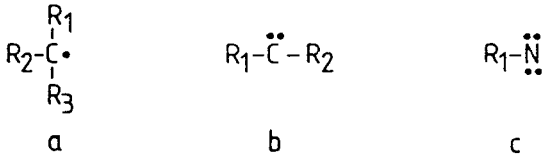


Fig 4. Highly reactive photogenerated intermediates: radical (a), carbene (b), nitrene (c).

directly insert into C-H bonds or first abstract a hydrogen atom from the C-H bond forming two radicals which may then combine to form the same product obtained by direct insertion. A fourth important reaction is the addition of carbenes to multiple bonds, yielding a three-member ring. Addition to an aromatic ring often results in a ring expansion. The reactions of carbenes are analogous to the reactions of nitrenes [cf. Fig 5].

A disadvantage of carbenes is their susceptibility for rearrangements to less reactive intermediates. Especially α-diazocarbonyl compounds form α-ketocarbenes upon irradiation which easily undergo the intramolecular Wolff rearrangement, yielding a ketene. Owing to the lower reactivity of these ketenes, the desired non-selectivity of the carbenes is lost. Ketenes react preferentially with nucleophilic amino acid residues.

Carbenes are formed upon irradiation of diazo compounds like α-diazoketones, aryldiazomethanes, α-diazoacetyl- and α-diazomalonyl-derivatives, or aryldiazirines. The aryldiazirines appear to be useful precursors for arylcarbenes because they are relatively stable in the dark and not very susceptible to intramolecular rearrangement after photolysis.

Nitrenes. Nitrenes, the nitrogen analogs of carbenes, were first applied to photoaffinity labeling of antibodies (Fleet *et al*, 1969). Their reactions are similar to those of carbenes [Fig 5]. Possible reactions of nitrenes include cycloadditions to multiple bonds forming three-member cyclic imines (reaction 1), addition to nucleophiles (reaction 2), direct insertion into C-H bonds yielding secondary amines (reaction 3), and hydrogen atom abstraction followed by coupling of the formed radicals to a secondary amine (reaactions 4a, b). The reactivity of nitrenes, however, is much lower than that of carbenes. They discriminate much more between primary, secondary and tertiary C-H bonds. In addition, nitrenes resemble electrophilic reagents in their reactivity, resulting in a preferential attack of 0-H, S-H, or N-H bonds over C-H bonds.

Nitrenes can be obtained by photolysis of several azido compounds. Alkyl azides, acyl azides, or aryl azides are possible precursors of nitrenes. Acyl and alkyl azides are not very suitable for photoaffinity labeling because of their instability, their susceptibility for rearrangements, and their improper absorption characteristics. Practical considerations favor aryl azides for photoaffinity labeling. Aryl azides possess three criteria which are ideal for an inactive precursor: they are chemically inert in the dark, the readiness of the generated reactive species to rearrange is very low, and they can be photolyzed at longer wavelengths ($\lambda > 300$ nm). Furthermore, the synthesis of aryl

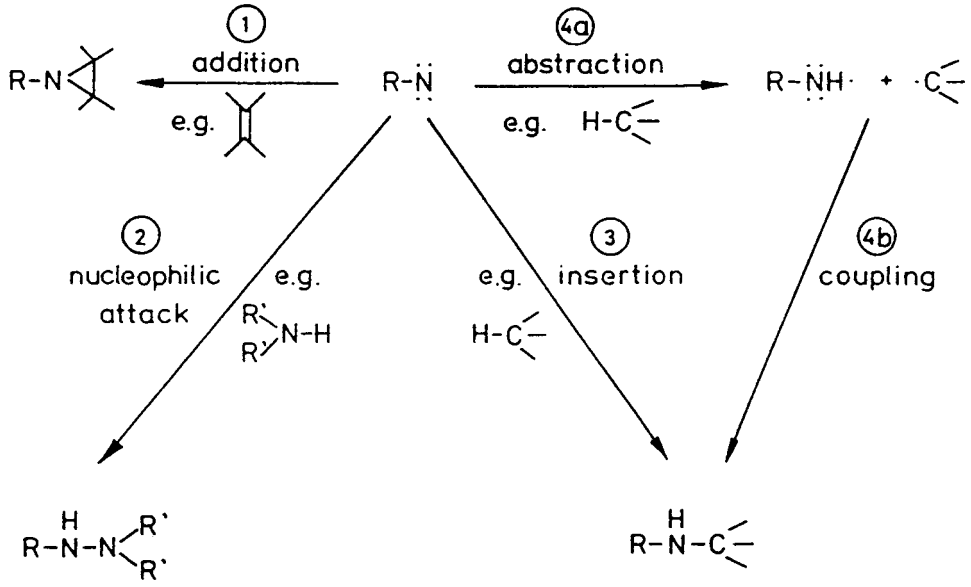


Fig 5. Reactions of nitrenes.

azides is relatively simple. Usually the corresponding aniline is diazotized and subsequently treated with sodium azide at -20°C. For these reasons aryl azides are the most commonly used precursors for photoaffinity labeling.

Free radicals and excited states. The third group of suitable precursors are those which form free radicals or excited states upon irradiation. The most frequently applied precursors of this class of reagents are α, ß-unsaturated ketones. Irradiation of α, ß-unsaturated ketones produces a diradical triplet state via the excited singlet. The diradical prefers to abstract a hydrogen atom from a C-H bond, resulting in the formation of two monoradicals which subsequently couple. 0-H bonds are much less attacked by the reactive intermediates. For this reason, radicals have advantages over nitrenes or carbenes, for these tend to react preferentially with the solvent water.

Besides the irradiation of α, ß-unsaturated ketones, the photoactivation of aryl halides, nitroaryl compounds, purines, pyrimidines, or psoralens can also produce free radicals and excited states.

Photoaffinity labeling, pseudophotoaffinity labeling, and non-specific photolabeling

Irradiation in the presence of a photoaffinity label can lead to the labeling of different regions of the receptor protein.

Ideally, the photoaffinity label first binds non-covalently at its binding site. Upon irradiation it forms a covalent bond to an amino acid at the binding site of the receptor protein (specific labeling).

In addition, the photoaffinity label is activated on the way to or from the specific binding site. In this case it labels the protein near the binding site (pseudoaffinity labeling). Pseudoaffinity labeling occurs also if the generated reactive intermediate possesses a longer lifetime. After its generation at the specific binding site, the intermediate dissociates from its place of origin and labels the protein away from this site.

Furthermore, the photoaffinity label may interact non-specifically with the receptor protein distant from its specific binding site. Upon irradiation it will label the protein at this site (non-specific labeling). Non-specific labeling may be important, especially in cases of extremely long lifetimes of the reactive intermediates.

Most of the photoaffinity label reacts after its activation with the solvent water. Normally this product can be easily separated from the labeled protein owing to its lower molecular mass.

The main problems of photoaffinity labeling are pseudophotoaffinity labeling and non-specific labeling. The proportion of pseudophotoaffinity labeling and non-specific labeling should be as low as possible. It is desirable to determine this portion quantitatively. The addition of scavengers reduces nonspecific labeling and pseudophotoaffinity labeling. A scavenger is capable of trapping reactive intermediates outside the ligand site (*i.e.*, those formed outside and those that dissociate from the binding site after their formation). There are two independent means of measuring the degree of non-specific labeling:

(1) A comparison of the results obtained by irradiation in the presence of the photoaffinity label and irradiation in the presence of a nonspecific interacting photoactivable reagent yields the proportions of specific and non-specific labeling. This reagent should possess, as much as possible, structural elements of the photoaffinity label and its photoactivable group. In contrast to the specific label, it should display no affinity for the ligand binding site of the receptor. It should therefore produce only non-specific labeling.

(2) The second way to discriminate between specific and non-specific labeling is by using a protecting reagent [Fig 6]. For this purpose, either the natural ligand or a non-reactive ligand analog is added in a labeling experiment. The photoaffinity label is displaced from the binding site by the ligand or its analog, resulting in the decrease, or ideally in the suppression, of specific labeling. The comparison between the unprotected and the protected labeling experiments indicates the degree of specific and non-specific labeling.

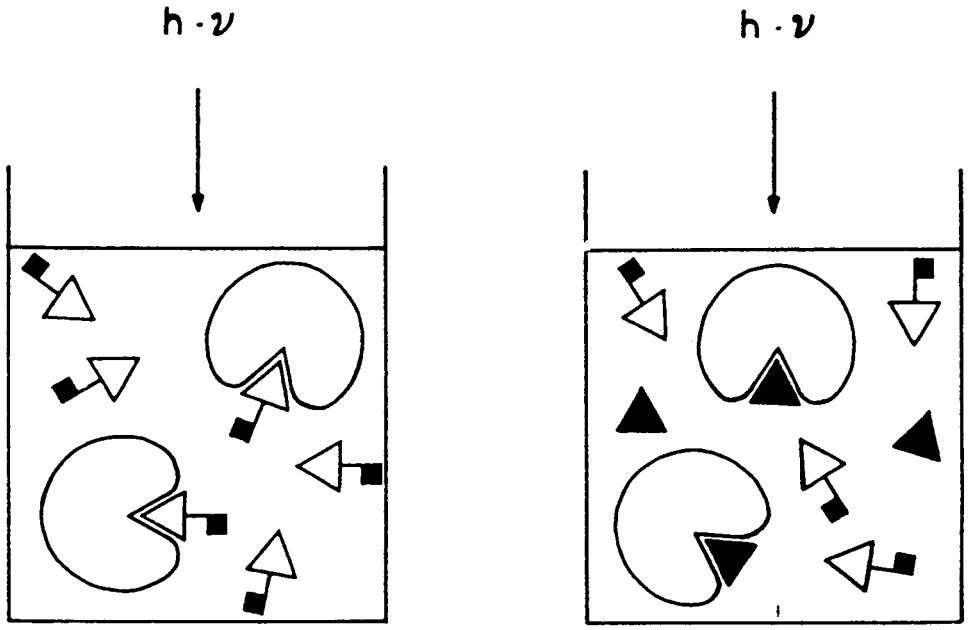


Fig 6. Unprotected (left) and protected (right) photoaffinity labeling experiment [photoaffinity label (open triangle); ligand analog(filled triangle)].

Examples of photoaffinity labeling

Photoaffinity labeling can be applied to the study of different problems. Three examples are given here.

Identification of a receptor in a mixture. Most hormone receptors are only present in small amounts in the plasma membrane among other proteins. These small amounts can be detected and isolated after photoaffinity labeling with radiolabeled photoactivable hormone analogs as demonstrated for the insulin receptor (Jacobs *et al*, 1979).

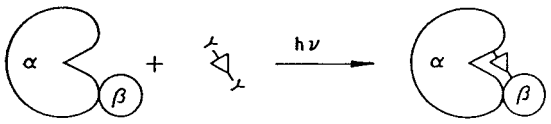
Identification of the ligand binding component of a multisubunit system. In multisubunit protein complexes the subunit containing a specific binding site can be identified by photoaffinity labeling. For example, the nucleotide binding sites of the ATP synthase complex (Hollemans *et al*, 1983; Vignais & Lunardi, 1985; Cross *et al*, 1987; Lunardi *et al*, 1987) and specific binding sites of ribosomal proteins have been photolabeled and thus characterized (Hsiung *et al*, 1974; Hsiung & Cantor, 1974).

Identification and characterization of a ligand binding site within a polypeptide. After photoaffinity labeling and degradation of the receptor protein by proteolysis or chemical cleavage, a small peptide containing the bound photoaffinity label can be isolated and precisely mapped. Ideally, only one amino acid of the ligand binding site should be labeled. Owing to the high reactivity of the intermediate, however, often several amino acid residues are labeled. 8-Azido ATP, for example, labels three different amino acids of the nucleotide binding site at the ß subunit of the mitochondrial F_1ATPase (Hollemans *et al*, 1983).

Photoaffinity crosslinking

The application of bifunctional reagents instead of monofunctional ones results in the crosslinking of proteins. This crosslinking may occur inter- or intramolecularly. The stabilization of the tertiary structure of proteins, the determination of distances between reactive groups in proteins, and the study of protein-protein interactions are useful applications of crosslinking reagents. A very important application of bifunctional reagents is for investigating the spatial arrangement of components in biological systems like membranes, ribosomes, or multisubunit enzymes. Crosslinking can also be achieved by reagents with one photosensitive and one conventional functional group, or with two photosensitive groups

(photo crosslinking). The introduction of two highly reactive functional groups into a biological ligand creates a tool for studying the vicinity of the specific binding site by crosslinking (affinity crosslinking). A biological ligand possessing two photoactivable groups forms a photoaffinity label capable of specifically crosslinking proteins upon light activation (photoaffinity crosslinking) [Fig 7] (Schäfer, 1986). Photoaffinity crosslinking possesses the same advantages over crosslinking or affinity crosslinking as photoaffinity labeling over protein modification by group-specific reagents or affinity labels. Affinity crosslinking and photoaffinity crosslinking have been proved to be very helpful, especially for the demonstration of an interfacial position of ligand binding sites. Irradiation of a receptor protein complex formed by different subunits in the presence of a bifunctional photoaffinity label should result in the crosslinking of subunits whenever the specific binding site is located at an interface between two subunits.



active inactive

Fig 7. Photoaffinity crosslinking of enzyme complexes (Y-Δ-Y : bifunctional photoaffinity label).

The photoaffinity labeling experiment

The following experimental sequence can be recommended.

Demonstration of the biological activity of the photoaffinity label. The most important precondition for a useful photoaffinity label is its biological activity. The photoaffinity label must show the same specific interactions with the receptor protein as the natural ligand. A suitable photoaffinity label for an enzyme should be a substrate (in the dark) or at least a competitive inhibitor.

Light-induced inactivation in presence of the photoaffinity label. Irradiation of the receptor protein in the presence of the photoaffinity label should decrease or even destroy the biological activity of the receptor protein. This inactivation should not be observed in the following control experiments: neither by incubation of the receptor with the photoaffinity label in the dark (dark control) nor by irradiation of the receptor protein in the absence of the photoaffinity label (light control).

Protection from light-induced inactivation by addition of natural ligands. The addition of the natural ligand or a non-reactive ligand analog should protect the receptor protein against the attack of the photoaffinity label, resulting in the maintenance of biological activity. Substrates, products, or competitive inhibitors are suitable protecting reagents for an enzyme.

Photoaffinity labeling with radiolabeled photoactivable ligand analogs. If the preceding experiments have been successful in demonstrating the specific interaction of the receptor protein with the photoaffinity label and the specific photoinactivation of the receptor protein, it is useful to employ a radiolabeled photoaffinity label. After the incorporation of the photoaffinity label, the degree of labeling can be measured, the labeled subunits of protein complexes can be identified, and essential amino acid residues or the sequence of the ligand binding site can be determined.

Protection from photoaffinity labeling. The addition of the natural ligand or a ligand analog should decrease or even prevent the labeling of the receptor protein in analogy to the protection from light-induced inactivation.

PHOTOAFFINITY LABELING AND PHOTOAFFINITY CROSSLINKING OF F_1ATPase FROM *Micrococcus luteus*

The second part of this article demonstrates selected examples of photoaffinity labeling and photoaffinity crosslinking performed in our laboratory. We have synthesized several monofunctional photoactivable adenine nucleotides - 2- and 8-azido ATP (2-N_3ATP, 8-N_3ATP) and the fluorescent 2- and 8-azido–1,N^6-etheno ATP (2-N_3εATP, 8-N_3εATP) [Fig 8] - to label and characterize the nucleotide binding sites of F_1ATPases from bacteria, mitochondria and chloroplasts (Schäfer *et al*, 1978a, 1978b, 1989a; Schuhen

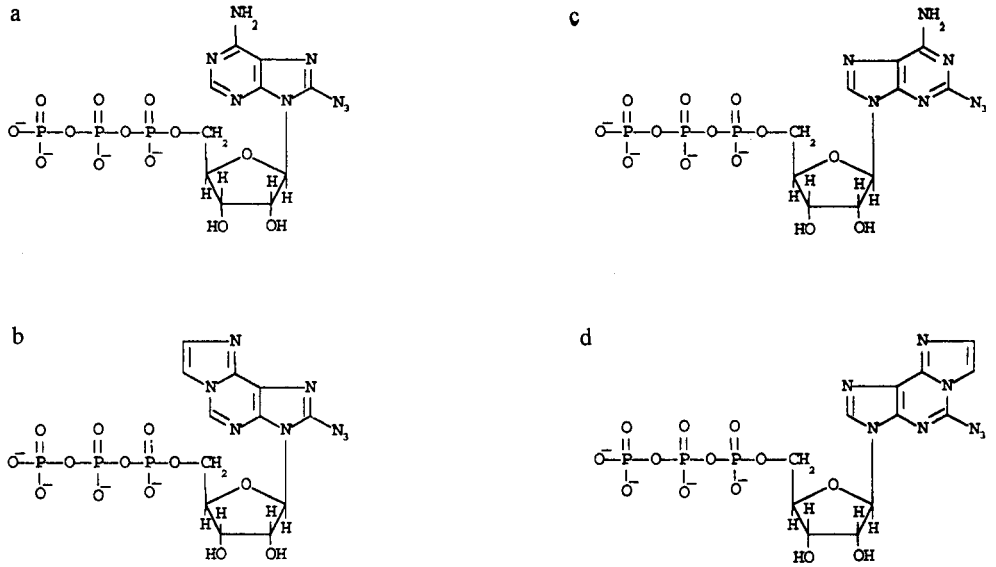


Fig 8. Monofunctional photoactivable ATP analogs: 8-N_3ATP (a); 8-N_3εATP (b); 2-N_3ATP (c); 2-N_3εATP (d).

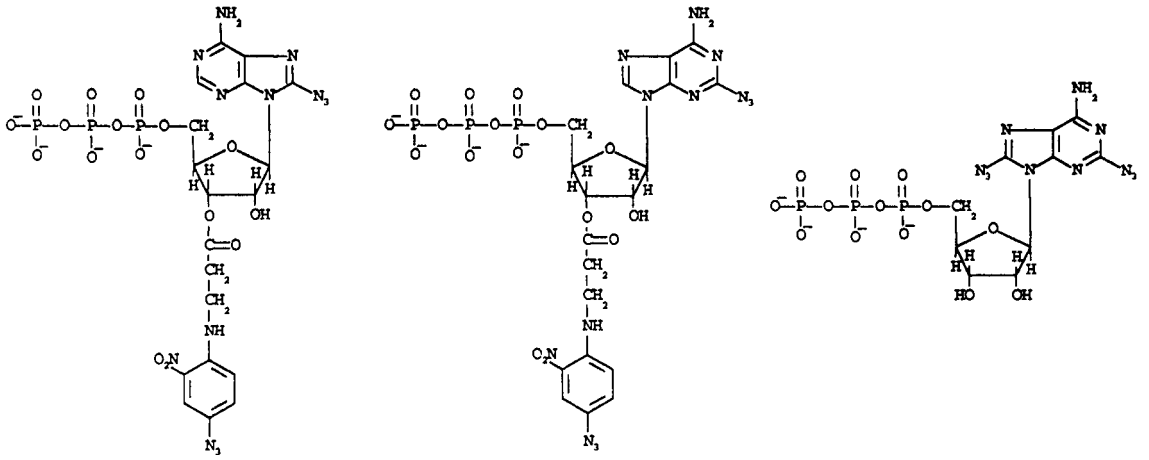
et al, 1994). The synthesis of various bifunctional (crosslinking) photoactivable adenine nucleotides - 3'-arylazido-ß-alanine-8-azido ATP (8,3'-DiN_3ATP), 3'-arylazido-ß-alanine-2-azido ATP (2,3'-DiN_3ATP) and 2,8-diazido ATP (2,8-DiN_3ATP) [Fig 9] - yielded useful tools for the investigation of the neighborhood of nucleotide binding sites of these enzymes (Schäfer *et al*, 1980a, 1989a; Rathgeber & Schäfer, 1989).

F_1ATPase is the catalytic portion of the ATP synthase complex [Fig 10] (Senior & Wise, 1983; Pedersen & Carafoli, 1987a, 1987b; Boyer, 1989, 1993; Pedersen & Amzel, 1993). This complex is the terminal enzyme in oxidative phosphorylation and in photophosphorylation. It uses the electrochemical potential energy of a proton gradient formed by the electron transporting protein complexes in the membranes of mitochondria, bacteria and chloroplasts to synthesize ATP from ADP and phosphate. The ATP synthase complex is generally composed of a water-soluble component (F_1ATPase), where the synthesis of ATP occurs, and a membrane-integrated part (F_0) which contains the proton-conducting channel of the ATP synthase. Solubilized F_1ATPase generally consists of five different subunits (α, β, γ, δ and ε). The stoichiometry of the F_1ATPases usually is α3β3γδε, containing three catalytic sites for ATP synthesis and ATP hydrolysis and three non-catalytic (probably regulatory) nucleotide binding sites. The composition of the F_0 part, however, differs widely depending on the origin of the ATP synthase complex.

The photoaffinity labeling and photoaffinity crosslinking experiments described here were mostly performed with a bacterial F_1ATPase from *Micrococcus luteus* and mono- and bifunctional 8-azido adenine nucleotides as photoaffinity labels (Scheurich *et al*, 1978; Schäfer *et al*, 1980b; Schäfer & Dose, 1984). The investigation of F_1ATPases from other origins (thermophilic bacteria PS3; mitochondria; chloroplasts) and the application of 2-azido adenine nucleotides as mono- and bifunctional photoaffinity labels yielded analogous results (Schäfer *et al*, 1983, 1984, 1985, 1989b, 1994; Schuhen *et al*, 1994).

Biological activity of 8-azido-adenosine nucleotides

The 8-substituted ATP analogs (8-N_3ATP, 8-N_3εATP, 8,3'-DiN_3ATP) are hydrolyzed by F_1ATPase from *Micrococcus luteus* in the presence of divalent metal ions. The rate of hydrolysis for these analogs is drastically decreased compared with the natural substrate ATP. This is due to the changed conformation of 8-substituted ATP analogs



a b c

Fig 9. Bifunctional photoactivable ATP analogs: 8,3'-DiN_3ATP (a); 2,3'-DiN_3ATP (b); 2,8-DiN_3ATP (c).

which prefer the syn conformation due to the bulky substituents at position 8 of the adenine ring. This is contrary to the preferred anti-conformation of ATP (Ikehara *et al*, 1972; Vignais & Lunardi, 1985) and 2-azido ATP (Czarnecki, 1984) [Fig 11]. On account of the very poor hydrolysis rate for 8,3'-DiN_3ATP, the biological activity of this divalent ATP analog is demonstrated by a second independent experiment [Fig 12], where the effect of 8,3'-DiN_3ATP on the hydrolysis of substrate [Ca•ATP] is determined. 8,3'-DiN_3ATP behaves as a competitive inhibitor, thus demonstrating the specific interaction between this modified nucleotide and the enzyme. All these 8-substituted ATP analogs are therefore suitable photoaffinity labels of F_1ATPases.

Light-induced inactivation of F_1ATPase by 8-N_3ATP analogs

Irradiation of the enzyme in the presence of a photoactivable 8-N_3ATP analog and Mg^{2+} ions results in an inhibition of ATPase activity [Fig 13]. This inactivation is observed neither by irradiation of the enzyme in the absence of photoaffinity labels (light control) nor by incubation of the enzyme with one of these labels (dark control).

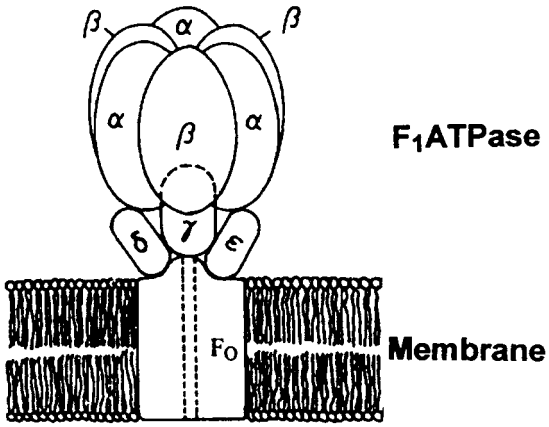


Fig 10. Structure of the ATP synthase complex.

Protection from light-induced inactivation of F_1ATPase by addition of ATP or ADP

The addition of ATP or ADP to the photoaffinity labeling experiment prior to irradiation protects the F_1ATPase from light-induced inhibition [Fig 14]. These nucleotides compete with the photoaffinity labels for nucleotide binding sites of the enzyme. AMP has no affinity to these binding sites; therefore its addition does not affect the inactivation by photoaffinity labeling.

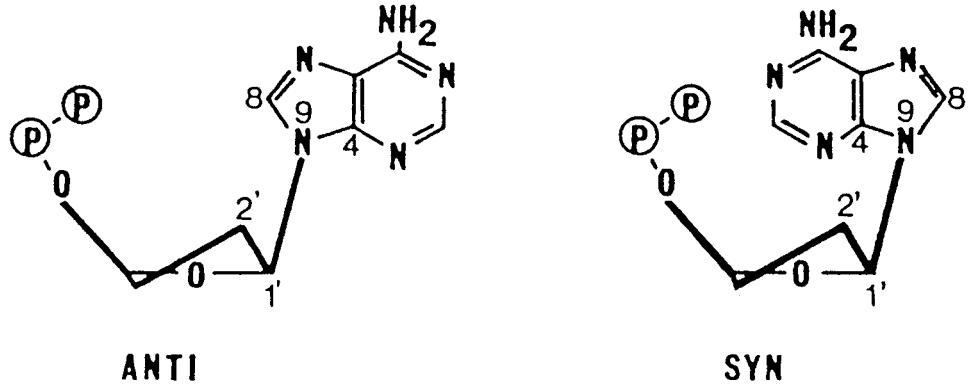


Fig 11. Conformations of adenine nucleotides (Vignais & Lunardi, 1985).

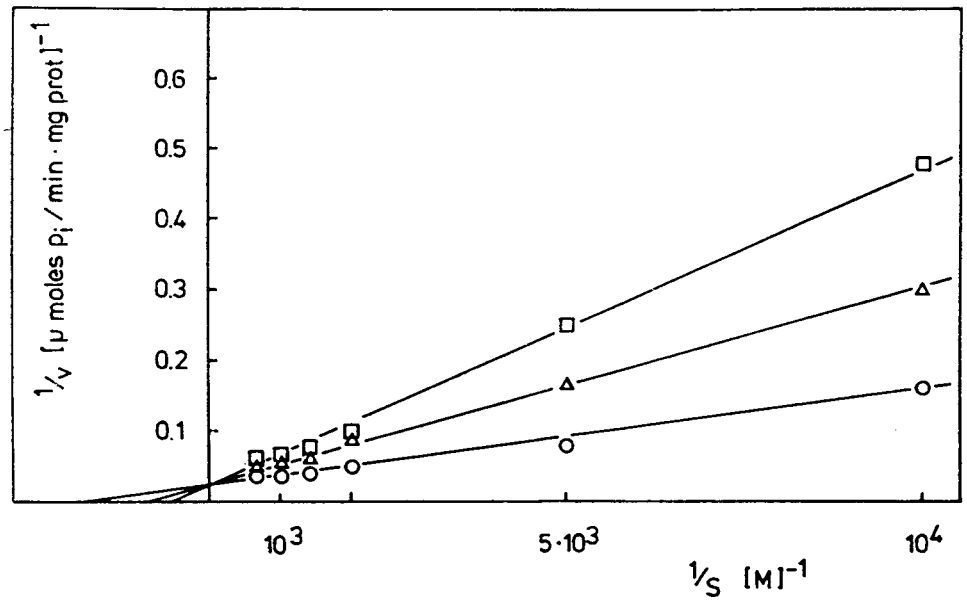


Fig 12. The effect of 8,3'-DiN_3ATP on the hydrolysis of ATP. Lineweaver-Burk plots (1/v vs 1/[CaATP] of F_1ATPase in the absence of 8,3'-DiN_3ATP (circles) and in the presence of 8,3'-DiN_3ATP [0.05 mm (triangles); 0.075 mM (squares)].

Photoaffinity labeling and photoaffinity crosslinking of F_1ATPase by monovalent 8-N_3ATP / 8-N_3εATP and divalent 8,3'-DiN_3ATP

F_1ATPase is photoinactivated in the presence of 8-N_3[^{14}C]ATP and Mg^{2+} ions. SDS gel electrophoresis of the labeled enzyme demonstrates that the main portion of the radiolabeled 8-N_3ATP is bound covalently to the b subunit [Fig 15]. After photoaffinity labeling with the fluorescent 8-N_3ATP and subsequent electrophoretic separation of the subunits, most of the fluorescence is observed at the position of the b subunit as well.

The irradiation of F_1ATPase in the presence of 8,3'-DiN_3ATP and Mg^{2+} ions results in the formation of crosslinked proteins. The SDS electrophoresis gel of the labeled enzyme shows an additional protein band with a molecular mass of about 120 kDa [Fig 16]. This band is not seen in control experiments with the unlabeled F_1ATPase, or with the enzyme labeled and inactivated by the monovalent 8-N_3ATP. The composition of the crosslink was elucidated by hydrolytic cleavage of the crosslink and

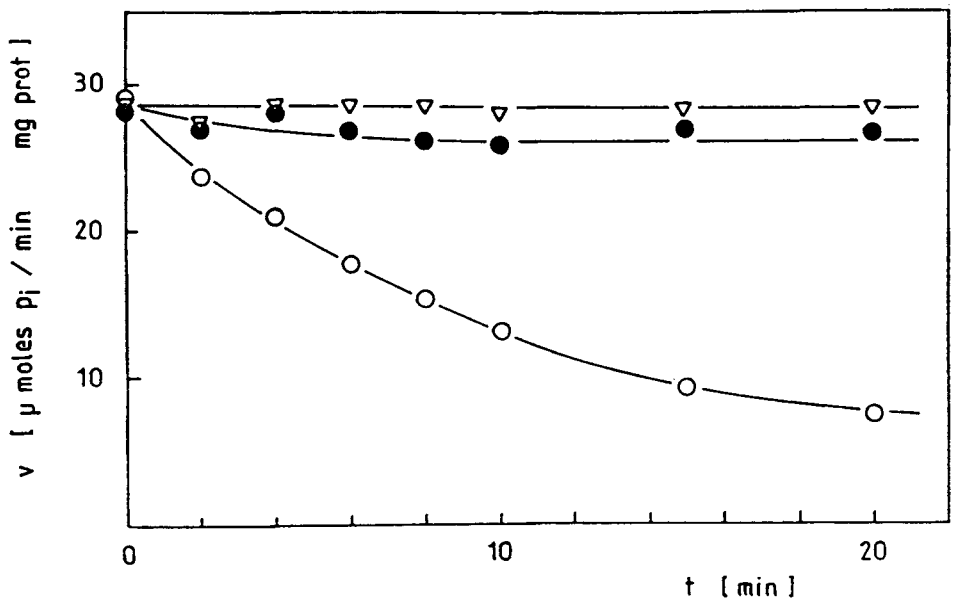


Fig 13. Light-induced inhibition of F_1ATPase. Irradiation in the presence of 0.5 mM Mg • 8,3' -DiN_3ATP (open circles); light control in the absence of 8,3'-DiN_3ATP (triangles); dark control in the presence of 0.5 mM Mg • 8,3'-DiN_3ATP (filled circles).

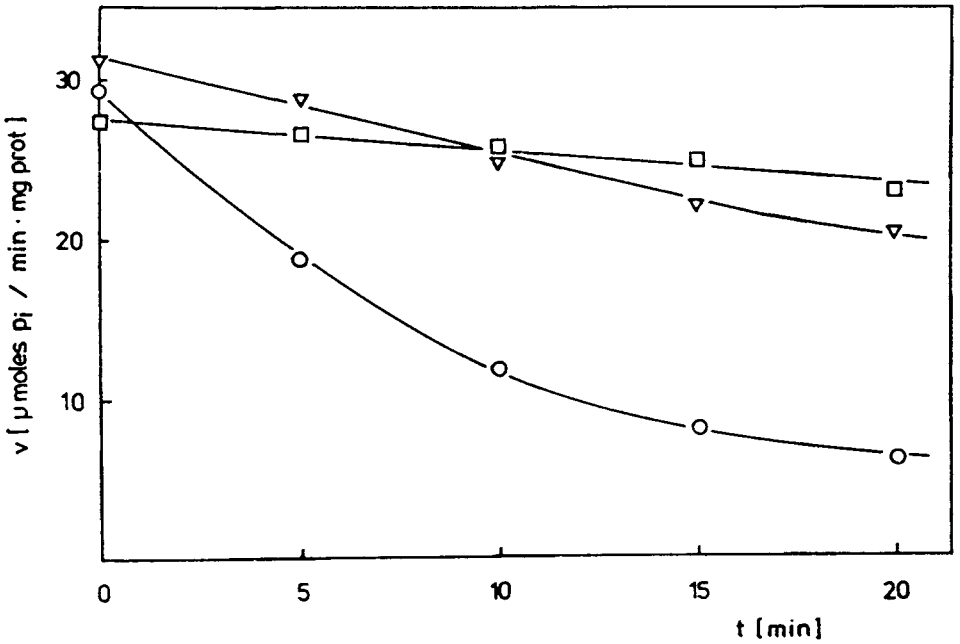


Fig 14. The effect of added Mg • nucleotides [1 mM Mg • AMP (circles); 1 mM Mg • ADP (triangles); 1 mM Mg • ATP (squares)] on the light-induced inhibition of F_1ATPase by 0.5 mM Mg • 8,3'-DiN_3ATP.

subsequent SDS gel electrophoresis [Fig 17]. The crosslink is almost entirely split into two protein bands of nearly identical concentration with a mobility corresponding to that of the a and b subunits. Independently, the crosslink's composition could be demonstrated by immunological techniques using specific antibodies against the purified subunits of F_1ATPase. These data indicate a subunit composition α-ß for the crosslink [Fig 18]. In some experiments - especially upon photoaffinity crosslinking of the F_1ATPase from the thermophilic bacterium PS3 or from chloroplasts, small amounts of

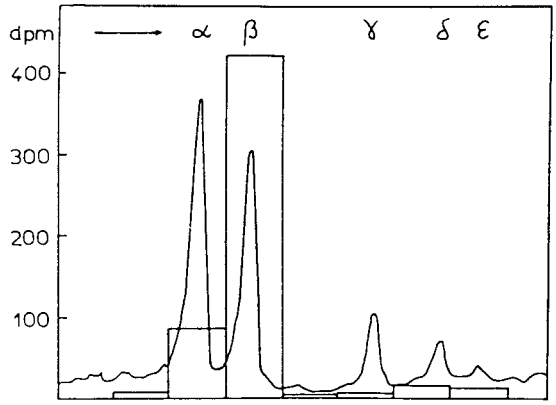


Fig 15. Photoaffinity labeling of F_1ATPase by 8-N_3[^{14}C]ATP.SDS gel electrophoresis densitogram of labeled F_1ATPase. The radioactivity of the gel slices is represented by the bars; the curve represents color density.

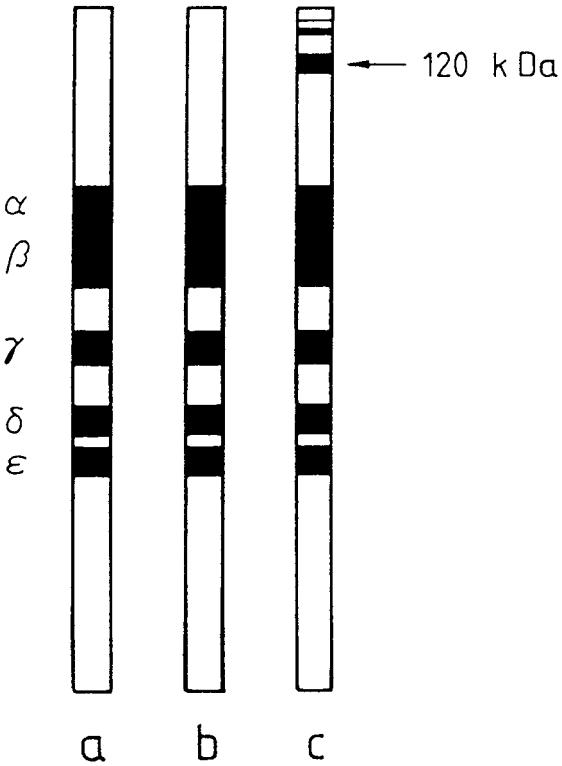


Fig 16. Photoaffinity crosslinking of F_1ATPase. SDS electrophoresis gels of labeled (crosslinked) F_1ATPase: native F_1ATPase (control) (a); F_1ATPase labeled by monovalent 0.5 mM Mg • 8-N_3ATP (b); F_1ATPase labeled by divalent 0.5 mM Mg • 8,3'-DiN_3ATP (c).

even higher molecular mass crosslinks were observed [cf. Fig 16c] besides the α-ß crosslink (120 kDa). These additional crosslinks are obviously formed by three [Fig 19] and four α and ß subunits, respectively.

Protection from photoaffinity labeling and photoaffinity crosslinking of F_1ATPase by addition of ATP or ADP

The addition of ATP or ADP prior to the labeling procedure protects the F_1ATPase from photoaffinity labeling with 8-N_3[^{14}C]ATP [Table I] or from photoaffinity crosslinking with 8,3'-DiN_3ATP. Both effects are not influenced by addition of AMP. These findings demonstrate that the hydrolytic nucleotide binding sites are specifically labeled and involved in the specific formation of the crosslinks. A further evidence for the specific labeling of the F_1ATPase by 8-N_3ATP is obtained by a control experiment with 8-N_3AMP. 8-N_3AMP does not interact specifically with the hydrolytic nucleotide binding sites of the enzyme. For this reason, 8-N_3AMP labels the F_1ATPase only non-specifically, and therefore the amount of labeling and light-induced inactivation is drastically reduced.

As a result of these experiments, it can be concluded that the catalytic nucleotide binding sites of F_1ATPase from Micrococcus luteus are located on the ß subunits very closely to the a subunits, probably at the interfaces between them. The formation of the three- and four-subunit crosslinks is in agreement with the position of all the catalytic and non-catalytic nucleotide binding sites of F_1ATPases at α-ß interfaces (Schäfer *et al*, 1985, 1989b, 1994) [Fig 20]. An interfacial position of all nucleotide binding sites presents an attractive model to explain the strong cooperativity between the different catalytic and noncatalytic (regulatory) sites postulated for the catalysis

Table I

Influence of various effectors on photoaffinity labeling of the and subunits with 8-N_3[^{14}C]AMP and 8-N_3[^{14}C]AMP.

Label	Effector	dpm		Inactivation
		a	ß	%
8-N_3ATP	Mg^{2+}	83	386	80
8-N_3ATP	Mg^{2+}, AMP	68	297	78
8-N_3ATP	Mg^{2+}, ADP	59	53	17
8-N_3ATP	Mg^{2+}, ATP	27	32	21
8-N_3AMP	Mg^{2+}	78	35	12

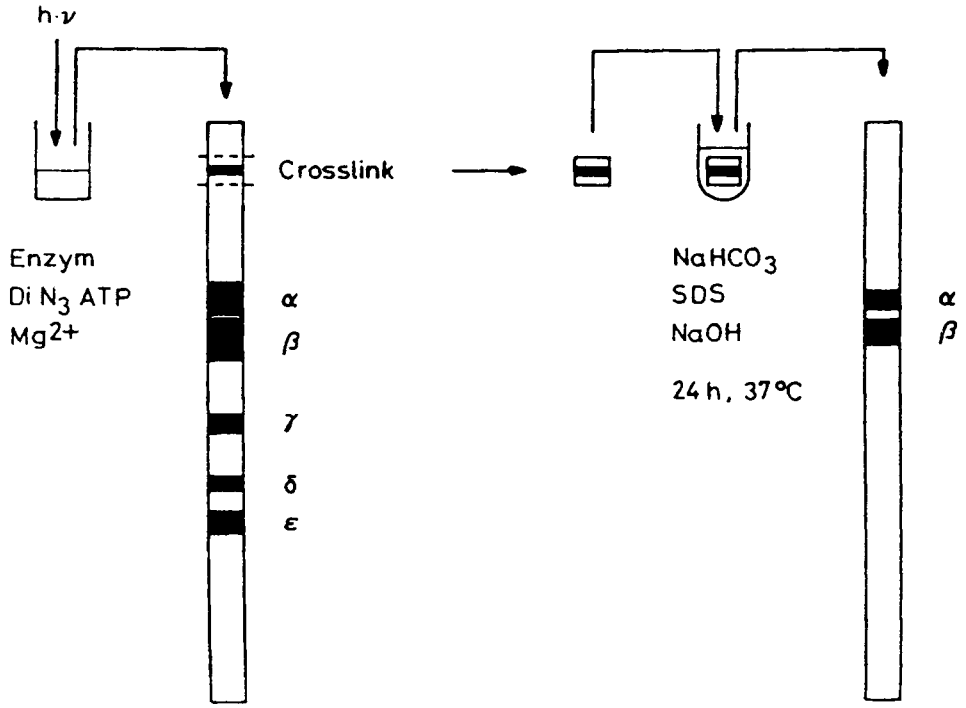


Fig 17. Hydrolytic cleavage of the crosslink.

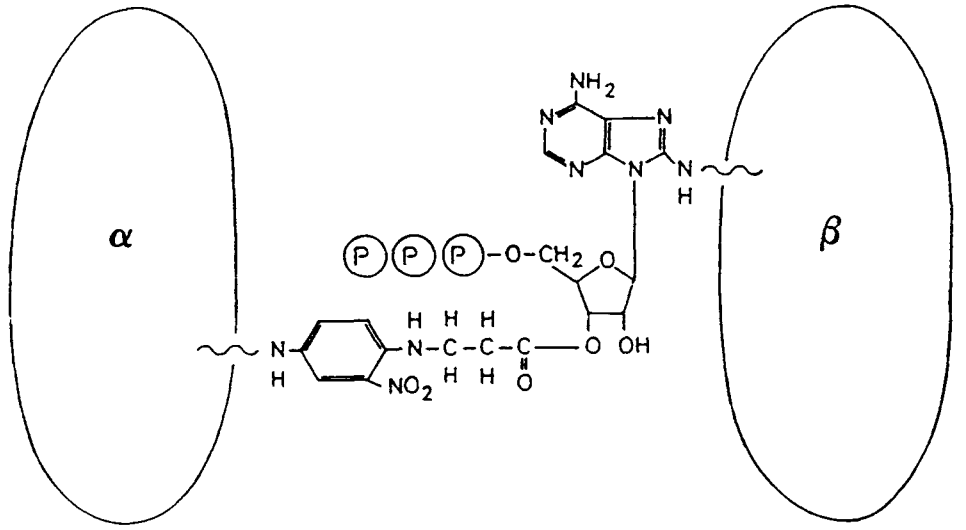


Fig 18. Structure of the α-ß crosslink.

performed by ATP synthases (alternating site mechanism; Boyer, 1989, 1993) [Fig 21]. Recently, this model could be confirmed by the determination of the structure of F_1ATPase from beef heart mitochondria by X-ray diffraction at 2.8 Å resolution (Abrahams *et al*, 1994). Up to this date, the photoaffinity crosslinking experiments were the only direct experimental approaches to prove the interfacial localization of nucleotide binding sites of ATP synthases.

Further characterization of the binding site can be performed, for example, by mapping the site and determining the nature of the labeled and crosslinked amino acid residues, as has been shown for the mitochondrial F_1ATPase (Hollemans *et al*, 1983; Cross *et al*, 1987; Lunardi *et al*, 1987; Zhou *et al*, 1992).

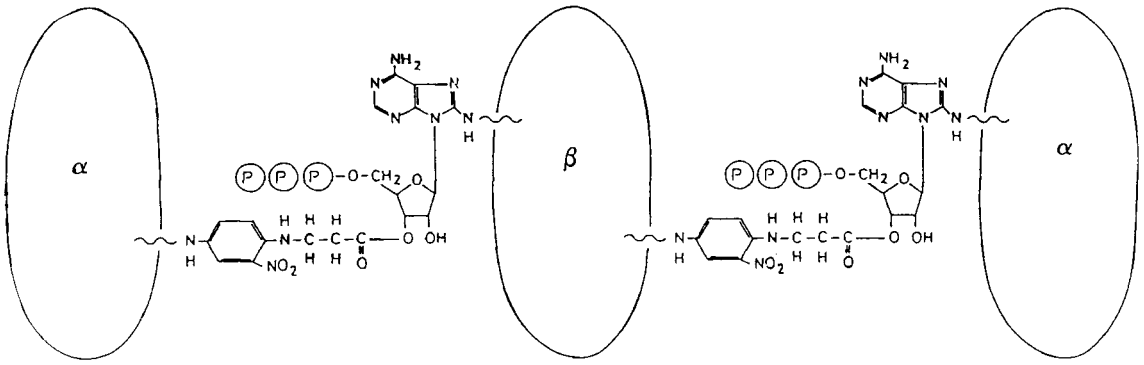


Fig 19. Possible structure of a crosslink formed by three α and ß subunits.

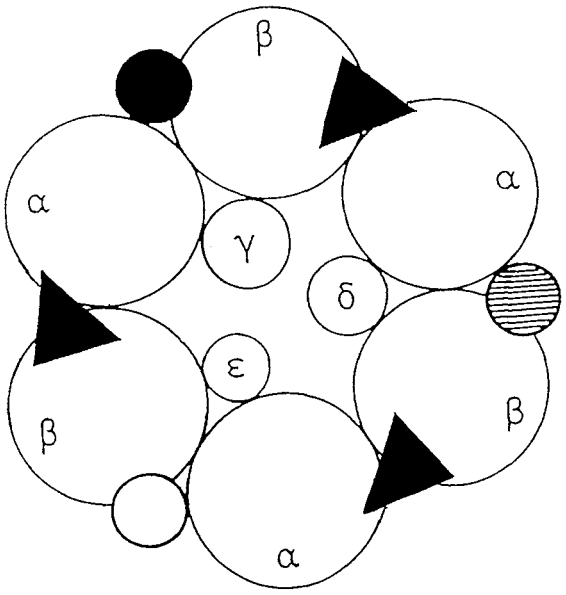


Fig 20. Position of the nucleotide binding sites of F_1ATPases [catalytic: open (O, open circles), loose (L, hatched circles), tight (T, filled circles); non-catalytic (triangles)].

Photoactivable diadenine dinucleotides

Recently, we have synthesized a new class of bifunctional photoactivable nucleotide analogs - diazido derivatives of diadenine dinucleotides [Fig 22]. Besides the introduction of 2- and 8-azido groups, additional fluorescent groups (etheno bridge or dansyl group) have been incorporated resulting in bifunctional photoactivable fluorescent diadenine dinucleotides (Schuhen *et al*, 1994). All these photoaffinity labels are very interesting for the investigation of receptor proteins possessing two nucleotide binding sites closely together like in adenylate kinases. Incubation and irradiation of e. g. adenylate kinases in the presence of these bifunctional dinucleotides should bridge and crosslink the different nucleotide binding sites.

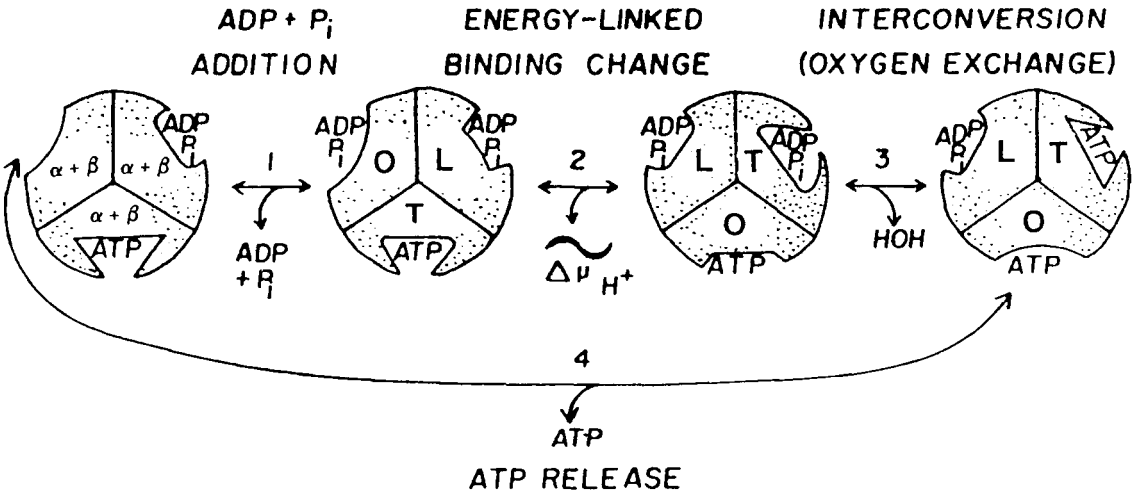
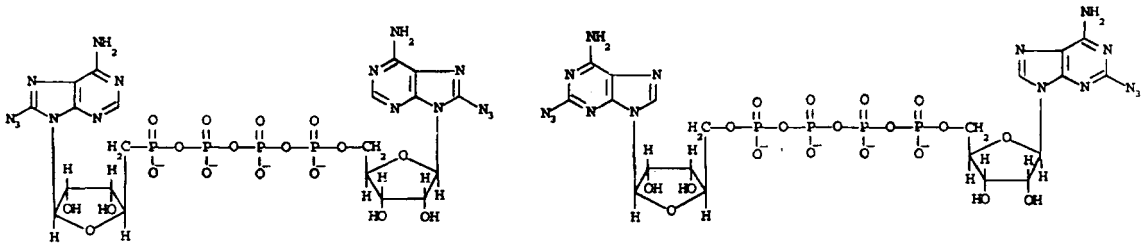


Fig 21. Alternating site mechanism for ATP synthetases [catalytic sites: open (O), loose (L), tight (T)]. (Boyer, 1989).



a b

Fig 22. Bifunctional photoactivable azido derivatives of diadenine dinucleotides: 8-N_3A-P_4-8-N_3A (a); 2-N_3A-P_4-2-N_3A (b).

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