Interaction of 2',3'-O-(2,4,6-Trinitriphenyl)adenosine Diphosphate and 2,4,6-Trinitrophenol with Thylakoid Membrane Proteins

Gudrun Ponse, Renate Thelen, and Heinrich Strotmann

Institut für Biochemie der Pflanzen, Heinrich Heine-Universität Düsseldorf, Universitätsstraße 1, D-W-4000 Düsseldorf, Bundesrepublik Deutschland

Z. Naturforsch. 47e, 264–274 (1992); received October 4, 1991

TNP-ADP, Picric Acid, Chloroplast, CF₀, CFᵣ-ATPase, Thylakoid Membrane

2',3'-O-(2,4,6-trinitrophenyl)adenosine-5'-diphosphate (TNP-ADP) is a competitive inhibitor of photophosphorylation of ADP. Here, the interaction of TNP-ADP with the H⁺-translocating chloroplast ATPase and other thylakoid membrane proteins was studied more in detail and compared with the interactions of the parental compounds ADP and 2,4,6-trinitrophenol (= picric acid).

The following results were obtained:

1. Preilluminated thylakoids incorporate about 1 nmol [14C]ADP per mg chlorophyll or the same amount of TNP-[14C]ADP in the following dark. If TNP-[14C]ADP is present in the light, however, up to 4 nmol label is bound per mg chlorophyll whereas the amount of incorporated [14C]ADP is unchanged. [14C]ADP is exclusively bound to CFᵣ, yielding a molar ratio of 1 nucleotide per enzyme. Likewise 1 mol of 14C label per mol enzyme is found in CFᵣ when thylakoids are illuminated in the presence of TNP-[14C]ADP; the residual bound radioactivity is related with other components of the thylakoid membrane.

2. TNP-ADP as well as picrate are electron acceptors of the photosynthetic electron transport chain. Picramate is one main product of photosynthetic reduction of picrate. Hence at least one of the three nitro groups of the trinitrophenyl moiety is reduced to an amino group. However, additional non-identified, reactive intermediates are formed, which can bind to membrane components. About 20% of the bound label is covalently linked to membrane proteins.

3. The patterns of covalently labeled polypeptides are identical whether [14C]picrate or TNP-[14C]ADP is employed, except for a and β subunits of CFᵣ, which are specifically labeled in the presence of TNP-[14C]ADP. This reaction is protected by (ADP + Pᵢ) > ATP > ADP > Pᵢ.

Most likely radicalic intermediates of picrate or TNP-ADP photoreduction are responsible for covalent binding. In case of CFᵣ the radicalic intermediate of TNP-ADP reduction may be accomodated by its ADP moiety into a nucleotide binding site. Subsequent covalent linkage via the reactive phenyl ring substituent seems to occur in both, the nucleotide and the Pᵢ binding domain.

Introduction

The use of adenine nucleotide analogues modified in the base or ribose moiety or in the phosphate chain, has contributed to the understanding of nucleotide interactions with the protein counterpart of nucleotide binding sites of the chloroplast H⁺-ATPase (review in ref. [1]). A variety of ADP and ATP analogues altered within the ribose moiety, turned out to be entire substrates in photophosphorylation or ATP hydrolysis, respectively. Thus 2'- or 3'-deoxidized or -methylated ADP could fully replace the parental compound ADP [2]. On the other hand, several 2'- or 3'-acylated ADP derivatives are competitive inhibitors of ADP photophosphorylation [3, 4]. These compounds show high affinity to the catalytic ADP binding site, but are very slowly phosphorylated. Less effective competitive inhibitors are the corresponding ATP derivatives. In the reverse reaction, i.e. in H⁺ transport-coupled ATP hydrolysis, however, the ATP derivatives are more inhibitory than the ADP analogues [5]. Based on the same chemical modification, photoaffinity labels have been synthesized (arylazido-ADP or -ATP) and used for localization of nucleotide binding sites within the ATPase complex [6].

Another type of useful ribose-modified nucleotide analogues are 2',3'-O-(2,4,6-trinitrophenyl)-
adenine nucleotides (TNP-ADP, TNP-ATP) because of their photophysical properties. These compounds exist either in 2'- or 3'-monoether form, or as a 2',3'-Meisenheimer complex depending on pH. As the pK is around 5, the Meisenheimer form is prevailing in solutions of physiological pH [7]. The fluorescent properties of TNP-adenine nucleotides have been utilized in studies of nucleotide interactions with isolated CF, [8], and for the determination by resonance energy transfer measurements [9] of intramolecular distances between nucleotide binding sites. Like 2'/3'-acylated ADP derivatives, TNP-ADP is a powerful competitive inhibitor of photophosphorylation [10].

The here reported finding that in illuminated chloroplast suspensions TNP-ADP is reduced by electrons from the photosynthetic electron transport chain, gave rise to a more detailed investigation of the interactions with CF, and other thylakoid membrane compounds. In illuminated chloroplasts non-covalent and unspecific covalent binding to membrane proteins due to the formation of reactive intermediates is demonstrated. A specific covalent linkage to α and β subunits of CF₁ requires the ADP moiety for recognition.

**Methods**

Envelope-free chloroplasts (= thylakoids) were isolated from spinach leaves as in ref. [11]. Binding of [¹⁴C]ADP and TNP-[¹⁴C]ADP to thylakoid membranes was measured as described in previous papers [11–13].

TNP-ADP was synthesized according to a method of Hiratsuka and Uchida [7]. Identity of the compound was ascertained by spectroscopic data from the literature [7, 14] and by phosphate analysis following total hydrolysis [15]. For synthesis of the ¹⁴C-labeled compound a small scale procedure was employed using TNBS and [⁸-¹⁴C]ADP as starting chemicals. The labeled TNP-ADP was isolated by preparative TLC on cellulose plates with n-butanol:n-propanol:water (2:1:1) as solvent. The TNP-[¹⁴C]ADP containing band was eluted by water. Purity and identity was examined by analytical TLC on PEI cellulose and spectroscopy [7, 14].

¹⁴C]picric acid was prepared by alkaline hydrolysis of [¹⁴C]TNBS for 24 h in LiOH at pH 9.0. The product was separated by preparative TLC as above. Identity was checked by co-chromatography with authentic unlabeled picric acid which was detected by its yellow colour. The concentrations of TNP-[¹⁴C]ADP and [¹⁴C]picric acid were determined by the known specific radioactivities of the labeled educts. Concentrations of unlabeled TNP-ADP were adjusted by optical density at 259, 408 and 470 nm using the extinction coefficients reported in ref. [7].

SDS electrophoresis of thylakoid membrane proteins was carried out on 15.5–22.5% gradient gels (acrylamide:bisacrylamide = 50:0.5) [16]. Labeled thylakoids were extracted by 80% acetone and pelleted by centrifugation. The precipitates were dissolved in a solution of 62.5 mM Tris buffer, pH 8.0, 2% SDS, 5% glycerol, 5% mercaptoethanol and 0.001% bromophenol blue and loaded onto the gels. After electrophoretic separation the protein bands were stained by Coomassie brilliant blue. Radioactive bands were detected by fluorography after treatment of the gels with scintillator Amplify (Packard) and exposure to X-ray films (Kodak-XAR 5) for 1 to 6 weeks. For quantitative determination of radioactivity the protein bands were excised from the gels, solubilized in H₂O₂/NH₃ [17] and measured in a scintillation counter.

CF₁CF₁ was isolated from labeled membranes by immunoprecipitation with anti-CF₁ following membrane solubilization by Triton X-100 [18].

**Results**

“Tight” binding of labeled ADP and TNP-ADP to pre-illuminated and illuminated thylakoid membranes

CF₁ of dark-adapted chloroplasts is known to contain a “tightly” bound nucleotide which is released or exchanged upon membrane energization [11–13]. The binding site is located in one of the three β subunits [19–21] and was identified as a catalytic nucleotide binding site [21]. The energy-linked release of the tightly bound nucleotide is a reaction related with the transfer of the enzyme from inactivity to catalytic activity [22–24]. The nucleotide depleted site generated by preillumination of thylakoids in the absence of medium nucleotides re-binds medium ADP added after de-energization [11]. ADP binding triggers deactivation of the thiol-modulated ATPase [22, 25]. On the other hand, illumination in the presence of me-
medium ADP, ADP + P_, or ATP effects fast exchange of bound against free nucleotides [24]. In the light the steady state level of ATPases containing a tightly bound nucleotide is inverse to the magnitude of ΔµH⁺ [26]. Upon deenergization, the whole enzyme population binds ADP within less than 1 min [27].

In an experiment shown in Fig. 1, thylakoids were either preilluminated and then provided with [14C]ADP or TNP-[14C]ADP, respectively, or illuminated in the presence of these nucleotides. Subsequently the membranes were analyzed for the contents of tightly bound label. The amounts of labeled nucleotides incorporated in the dark after preillumination were virtually identical with [14C]ADP or TNP-[14C]ADP, and the same amount of radioactivity was also bound when [14C]ADP was present during illumination. In all three cases binding was saturated at nucleotide concentrations < 5 \text{µM} and the maximal amounts of bound label were about 1 nmol/mg chlorophyll. In contrast, an up to 4-fold amount of radioactivity was incorporated when thylakoids were illuminated in the presence of TNP-[14C]ADP. In this case the concentration curve was biphasic; saturation of the low affinity phase was attained at TNP-[14C]ADP concentrations > 20 \text{µM}.

With thylakoids preloaded with either [14C]ADP or TNP-[14C]ADP in the light, the kinetics of light-induced release of labeled nucleotides were compared in a medium containing ADP + P_. Fig. 2a shows virtually identical initial rates of release; the final amount of radioactivity released from TNP-[14C]ADP-prelabeled membranes, however, was significantly lower, although the total amount of membrane-associated radioactivity was 1.5 times higher in this experiment than the amount of radioactivity present in the [14C]ADP-prelabeled thylakoids. More than half of the labeled nucleotides incorporated from TNP-[14C]ADP were not released, whereas all of the tightly bound [14C]ADP is liberated within a light period of 8 s (Fig. 2b).

In order to elicit the location of the bound label, CF₁ was isolated from thylakoids preloaded with TNP-[14C]ADP or [14C]ADP in the light and subsequently analyzed for the contents of radioactive nucleotides [11]. Table I compares the total amounts of bound radioactivity and the proportions of radioactivity related with CF₁. As expected from earlier results [11], all of the bound [14C]ADP was detected in CF₁, the molar ratio being about one nucleotide per enzyme. Preloading with TNP-[14C]ADP likewise yielded one labeled nucleotide bound per one enzyme molecule, indicating that the surplus radioactivity was either lost during solubilization of CF₁ or retained by the membrane fraction. Analysis of TNP-[14C]ADP-preloaded thylakoids after complete removal of CF₁ by NaBr treatment [28] indeed yielded a significant proportion of radioactivity related with the membranes whereas the [14C]ADP-preloaded and the CF₁-stripped membranes were virtually free from radioactivity (Table II). Almost no label was detected in both, the intact and CF₁-stripped membranes after pretreatment of thylakoids in the dark with either [14C]ADP or TNP-[14C]ADP.

**Photochemical reduction of TNP-ADP and picrate catalyzed by thylakoid membranes**

The preceding results demonstrate that TNP-ADP can replace the parental compound ADP in tight binding to the CF₁ sector of the ATPase.
situ. This is further substantiated by their mutual displacement in competition experiments (not shown). The finding that additional binding occurs with TNP-ADP only in light, suggests that a reaction product formed under these conditions rather than TNP-ADP itself is the reacting compound. A photochemical conversion of TNP-ADP is excluded since in the absence of chloroplasts, TNP-ADP is stable even towards illumination with white light. On the other hand, when chloroplasts are present, illumination with red light (>630 nm) which is not absorbed by TNP-ADP, yielded the same incorporation into the membranes as illumination with white light suggesting

![Graph](image)

Table I. Proportions of tightly bound [14C]ADP and TNP-[14C]ADP related with CF$_r$. Washed isolated thylakoids (1 mg chlorophyll ml$^{-1}$) were illuminated for 3 min in the presence of either 5 μM [14C]ADP or 5 μM TNP-[14C]ADP. After 10 min in the dark, free labeled nucleotides were removed by three subsequent washes. In aliquots the contents of 14C were determined. The residual pellets were resuspended in a medium containing 1 mm Tricine and 0.5 mm EDTA, pH 8.0, a treatment which extracts 40-60% of CF$_r$ in almost pure form [11]. The exact percentage of CF$_r$ released was determined from the methanol-stimulated ATPase activity [43] of the thylakoid membranes before and after EDTA treatment. From the protein contents of the supernatants and the percentage of released CF$_r$ the total amount of CF$_r$ was calculated as 0.42 mg protein/mg chlorophyll or 1.049 nmol CF$_r$/mg chlorophyll using the CF$_r$ molecular weight of 400 kDa [44]. The amounts of released labeled nucleotides were determined by measurements of 14C in the supernatants and related to the CF$_r$ protein contents.

<table>
<thead>
<tr>
<th>Reaction performed with</th>
<th>[14C]ADP</th>
<th>TNP-[14C]ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td>14C contents of the membranes before EDTA treatment [nmol/mg chl]</td>
<td>1.10</td>
<td>1.71</td>
</tr>
<tr>
<td>14C contents of the supernatants after EDTA treatment [nmol/mg protein]</td>
<td>2.72</td>
<td>2.69</td>
</tr>
<tr>
<td>[14C]nucleotides related with CF$_r$ [nmol/mg chl]</td>
<td>1.14</td>
<td>1.12</td>
</tr>
<tr>
<td>[mol/mol CF$_r$]</td>
<td>1.09</td>
<td>1.07</td>
</tr>
</tbody>
</table>
Table II. Proportion of bound [14C]ADP and TNP-[14C]ADP related with the thylakoid membrane. Washed thylakoids (1 mg chlorophyll/ml) were illuminated for 2 min in the presence of either 5 μM [14C]ADP or 5 μM TNP-[14C]ADP or kept in the dark. Free labeled nucleotides were removed by washing. Subsequently CF4 was extracted by 2 M NaBr [28]. The 14C contents of the membranes before and after NaBr treatment were determined.

<table>
<thead>
<tr>
<th>Bound [14C]nucleotides [nmol/mg chl]</th>
<th>Control</th>
<th>After NaBr treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>[14C]ADP light</td>
<td>0.69</td>
<td>0.01</td>
</tr>
<tr>
<td>dark</td>
<td>0.04</td>
<td>0.01</td>
</tr>
<tr>
<td>TNP-[14C]ADP light</td>
<td>1.45</td>
<td>0.55</td>
</tr>
<tr>
<td>dark</td>
<td>0.10</td>
<td>0.02</td>
</tr>
</tbody>
</table>

As TNP-ADP is a nucleotide derivative of trinitrophenol (= picric acid) analogous experiments were conducted with [14C]TNBS and [14C]picrate. Analysis by two different TLC systems likewise showed formation of several radioactive products at the expense of the labeled educts. One of the compounds was identified by co-chromatography with the authentic compound as [14C]picramate (Table III). The formation of picramate from picrate includes reduction of one of the three nitro groups to an amino group, a process which requires uptake of altogether 6 electrons. As the reduction most likely proceeds in several single or paired electron transfer steps, formation of unstable or metastable reactive intermediates may be supposed (see Discussion). Moreover reduction of the second and third nitro groups must be taken into account, i.e. photosynthetic reduction of trinitrophenyl compounds is a rather complex process. The degradation of TNP-ADP or picric acid, respectively, is inhibited by phenazine methosulfate, a mediator of PSI-cyclic electron transport (Table IV). Hence the trinitrophenyl compounds are most probably electron acceptors of photosystem I.

TNP-ADP as electron acceptor of the photosynthetic electron transport chain was further studied by oxygen measurements (Fig. 4). Illumination of chloroplasts in a basal medium containing buffer, MgCl2 and NaCl, effects slow oxygen uptake due to a Mehler reaction. Upon addition of TNP-ADP, initial oxygen uptake is accelerated up to 5-fold. Thus TNP-ADP resembles methylviologen, an electron acceptor which is continuously regenerated since the reduced form undergoes reoxi-
Table III. \( R_f \) values of reaction products formed from TNBS and picrate, respectively, by illumination of isolated chloroplasts. Isolated chloroplasts (0.33 mg chlorophyll/ml) were illuminated for 2 min in the presence of either 10 \( \mu \text{M} [{}^{14}\text{C}]\text{TNBS} \) or 10 \( \mu \text{M} [{}^{14}\text{C}]\text{picrate} \). The supernatants after centrifugation were subjected to TLC together with authentic TNBS, picrate and picramate. The radioactive spots were detected by autoradiography. In (A) cellulose-coated plates were employed; the chromatogram was developed in butanol:propanol:water (2:1:1). The PEI cellulose plates used in (B) were developed in 1 M LiCl plus 1 M formic acid.

(A) Chromatography on cellulose

<table>
<thead>
<tr>
<th>Starting compound:</th>
<th>Reaction products:</th>
<th>( R_f )</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNBS</td>
<td>picramate</td>
<td>0.68</td>
</tr>
<tr>
<td>picrate</td>
<td>( x_1 )</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>( x_2 )</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>start</td>
<td>0.00</td>
</tr>
</tbody>
</table>

(B) Chromatography on PEI cellulose

<table>
<thead>
<tr>
<th>Starting compound:</th>
<th>Reaction products:</th>
<th>( R_f )</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNBS</td>
<td>picramate</td>
<td>0.43</td>
</tr>
<tr>
<td>picrate</td>
<td>( y_1 )</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>picramate</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>( y_2 )</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>( y_3 )</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>( y_4 )</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>start</td>
<td>0.00</td>
</tr>
</tbody>
</table>

dation by molecular oxygen accompanied by the formation of hydrogen peroxide. In contrast to methylviologen, however, the rate of TNP-ADP-dependent oxygen consumption decreases with time, suggesting that the autoxidable reduced substance (or substances) are intermediates, whereas the final product may be stable towards oxidation by molecular oxygen. The partial non-cyclic and partial cyclic reaction of TNP-ADP in photosynthetic electron transport excludes determination of the actual electron stoichiometry. In accordance with its property as electron acceptor or mediator, respectively, TNP-ADP is capable of supporting light-induced generation of a transmembrane proton gradient (Fig. 5).

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Table IV. Photosynthetic reduction of TNP-[\(^{14}\text{C}\)]ADP in the absence or presence of 50 \( \mu \text{M} \) PMS. The TNP-[\(^{14}\text{C}\)]ADP concentration was 4 \( \mu \text{M} \), the chlorophyll concentration 0.36 mg/ml.

<table>
<thead>
<tr>
<th>Illumination time ([\text{s}])</th>
<th>Concentration of medium TNP-[(^{14}\text{C})]ADP ([\mu\text{M}])</th>
<th>+PMS</th>
<th>-PMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.0</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>3.6</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>3.2</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>1.8</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>300 (+10 ( \mu \text{M} ) DCMU)</td>
<td></td>
<td>4.0</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 5. Light-dependent formation of a transmembrane proton gradient induced by TNP-ADP as measured by 9-aminoacridine fluorescence quenching. The chlorophyll concentration was 34 μg ml⁻¹, the concentration of 9-aminoacridine (9-AA) 5 μM and the concentration of TNP-ADP 0.5 μM. The steady state ΔpH induced by Mehler reaction was 2.27, the maximal ΔpH obtained in the presence of TNP-ADP was 3.19. For calculation the formula given in ref. [42] and an internal thylakoid volume of 30 μl mg chlorophyll⁻¹ [26] was employed.

Covalent binding of reduction products of TNP-ADP and picrate

Some of the supposed metastable intermediates formed during photosynthetic reduction of TNP-ADP and picrate, respectively, might be linked covalently to polypeptides of the thylakoid membrane. To investigate this, thylakoids prelabeled with TNP-[¹⁴C]ADP or [¹⁴C]picrate in the light, were subjected to quantitative and qualitative analysis of covalently bound radioactivity. Fig. 6 shows that about 20% of the radioactivity incorporated from TNP-[¹⁴C]ADP or [¹⁴C]picric acid is indeed covalently linked to thylakoid membrane polypeptides. Covalent labeling as function of illumination time resembles the time courses of degradation of the starting compounds by photosynthetic reduction.

SDS electrophoresis followed by fluorography reveals almost identical labeling patterns of a variety of polypeptides ranging from 10 to 40 kDa with both, radioactive TNP-ADP and picric acid. However, incorporation of label into two bands in the 50–55 kDa range is observed in TNP-[¹⁴C]ADP-treated membranes but not in thylakoids illuminated with [¹⁴C]picric acid (Fig. 7). From TNP-[¹⁴C]ADP-prelabeled membranes the ATPase complex (CF₀CF₁) was isolated by immunoprecipitation using a monospecific polyclonal antibody raised against CF₁. The following fluorography of the SDS gel clearly identifies the labeled polypeptides as α and β subunits of CF₁. Some radioactivity observed in γ and in subunit I of CF₀ was not specific for TNP-[¹⁴C]ADP but was found with [¹⁴C]picric acid, too (not shown). TNP-[¹⁴C]ADP incubation of illuminated thylakoids which were pretreated with NaBr to remove CF₁ [28], likewise did not result in labeling of the two 50–55 kDa bands.
Table V gives a quantification of $^{14}$C incorporation into $\alpha$ and $\beta$ subunits during illumination of thylakoids with 10 $\mu$M TNP-$[^{14}$C]ADP for 2 min. In parallel experiments 5 mM $P_i$, 0.5 mM ADP, $P_i$ + ADP or 0.5 mM ATP was simultaneously added. The controls show an even distribution of radioactivity between the two subunits. Incorporation of 20 pmol per mg chl means that about 10 per cent of the total covalently bound radioactivity is located in the $CF_0$,$CF_1$ complex. The fact that ADP and ATP largely abolish covalent binding to $\alpha$ and $\beta$, indicates specific interactions with nucleotide binding sites. Most efficient protection, however, is observed with ADP + $P_i$. The finding that even $P_i$ alone effects significant reduction of covalent binding, particularly in $\alpha$ subunit, also suggests interaction of the nucleotide analogue with a phosphate binding site.

Table V. Incorporation of radioactivity into $\alpha$- and $\beta$-subunits of $CF_1$ during illumination of thylakoids with TNP-$[^{14}$C]ADP. Thylakoids (0.2 mg chlorophyll/ml) were illuminated for 2 min in a medium containing 25 mM tricine, pH 8.0, 50 mM NaCl, 5 mM MgCl$_2$, 10 $\mu$M TNP-$[^{14}$C]ADP, and phosphate (5 mM), ADP or ATP (0.5 mM) as indicated. After centrifugation, the pellets were dissolved in SDS-containing buffer and subjected to SDS-PAGE. After staining the $\alpha$- and $\beta$-bands were excised, dissolved in H$_2$O$_2$ containing 0.25% NH$_3$ (12 h at 37 °C) and counted in scintillator cocktail.

<table>
<thead>
<tr>
<th></th>
<th>$^{14}$C contents in $\alpha$</th>
<th></th>
<th>$^{14}$C contents in $\beta$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[pmol/mg chl]</td>
<td>[%]</td>
<td>[pmol/mg chl]</td>
</tr>
<tr>
<td>Control</td>
<td>10.2</td>
<td>100</td>
<td>10.5</td>
</tr>
<tr>
<td>+$P_i$</td>
<td>5.2</td>
<td>51</td>
<td>7.9</td>
</tr>
<tr>
<td>+ADP</td>
<td>4.2</td>
<td>41</td>
<td>5.4</td>
</tr>
<tr>
<td>+$P_i$ + ADP</td>
<td>2.6</td>
<td>25</td>
<td>4.1</td>
</tr>
<tr>
<td>+ATP</td>
<td>3.8</td>
<td>37</td>
<td>4.4</td>
</tr>
</tbody>
</table>

Discussion

According to a widely held view, three catalytic and three non-catalytic nucleotide binding sites are present per $CF_1$, one of each in one $\beta$ subunit [21], possibly in close vicinity to the $\alpha$ subunits [6]. The function of the non-catalytic sites which are permanently occupied by nucleotides in the ATPase in situ [29], is obscure. They were discriminated from catalytic sites by covalent labeling with 2-azido-ATP which binds at a tyrosyl residues located about 20 amino acids apart from a tyrosyl residue involved in catalytic nucleotide binding [21].

In the inactive state of the membrane-associated enzyme the three catalytic sites exhibit asymmetry as one of them is containing an occluded ("tightly bound") adenine nucleotide, notably ADP [11, 13, 19, 20, 24, 26, 30], whereas the other two sites are likewise inaccessible but empty [13, 31]. The tightly bound nucleotide is released upon activation by membrane energization and the vacant sites get available, too [24, 31]. In the working state of the enzyme all three sites may be involved in catalysis in a sequential mode as suggested by Boyer and his colleagues [32], however, participation of less than the three sites has also been proposed [33–36]. Deactivation of the ATPase following membrane deenergization is related with re-incorporation of a nucleotide molecule [22, 26].

In this paper it was demonstrated that TNP-ADP can replace ADP in tight binding to $CF_1$. In previous work [10] we have demonstrated the competitive inhibition of ADP phosphorylation by TNP-ADP indicating interaction of the ADP analogue with catalytic sites. In those studies brief illumination times of maximally 15 s were employed. As shown in the present paper, extensive photosynthetic reduction of TNP-ADP takes place at longer illumination times, in particular in the ab-
The related compounds TNBS and picrate are likewise electron acceptors of the photosynthetic electron transport chain, indicating that the trinitrophenyl ring is the target for electron abstraction. Reduction of one or more of the three nitro groups, most likely is a multistep electron transfer reaction, similar to the electrochemical reduction of nitrobenzene to aniline [37]. A hypothetic reaction mechanism for the reduction of a single nitro group is figured out in Scheme 1. The putative radicalic intermediates are predestinated to form covalent bonds with proteins, whereas the charged intermediates may undergo non-covalent binding to proteins via ionic interactions with oppositely charged amino acid side groups. During photosynthetic reduction, transitory accumulation of single intermediates appears possible, the more since the redox potential difference in Hill reaction decreases with each electron transfer step. Actually the occurrence of a variety of compounds could be demonstrated by chromatography. Because of their reactivity in covalent bond formation, further reduction and ready reoxidation by oxygen, however, the steady state concentrations of intermediate compounds are probably low.

Although covalent binding of the supposed radicalic intermediates are unselective, certain membrane proteins were preferentially attacked by reactive intermediates of picrate as well as TNP-ADP reduction. The accessible polypeptides most probably are extrinsic stroma-exposed proteins. The specific labeling of the α and β subunits of CF₁ by the reaction products of TNP-ADP but not of picrate, suggests that the ADP moiety of this molecule is necessary to guide the compound into a nucleotide binding site. Hence unreacted TNP-ADP as well as any intermediate or product of TNP-ADP reduction can undergo non-covalent binding to those sites. The formation of covalent bonds in a secondary reaction is restricted to the reactive species among the intermediates. In fact the proportion of covalently linked nucleotide analogues is low (Table V) while the bulk is reversibly bound (Table I, Fig. 2).

The recognition domains of the ADP molecule with regard to the active site of CF₁ are located in the amidino region including N-1, C-6 and the exocyclic amino group of the adenine base on one hand [38], and the phosphate chain on the other hand [39]. Photoaffinity labeling with the ADP analogue 2-azido-ADP has shown that the adenine binding domain is directed to a peptide strand including Tyr-362 of β subunit [21]. Although not finally substantiated, there is some indication that α-phosphate of ADP interacts with a specific lysyl residue of β subunit [40]. Accordingly, the ADP binding domain of the catalytic center seems to be located in β subunit. Compared with ADP, the recognition sites located in the adenine moiety and the phosphate chain are unchanged in TNP-ADP and its reduction products, respectively. Therefore a correct accommodation of these ADP analogues within a nucleotide binding site can be assumed. In its Meisenheimer structure TNP-ADP forms a rigid three-ring system with the trinitrophenyl ring perpendicular to the ribose ring. In contrast, the 2'- or 3'-monoether form permits rotational mobility of the trinitrophenyl ring relative to the ADP moiety. Although the employed medium pH (8.0)

![Scheme 1. Proposed reaction mechanism of photosynthetic reduction of nitrophenyl compounds.](image-url)
favours the Meisenheimer structure in solution, formation of monoether structure is not unlikely when the compound is bound to a protein. Upon more drastic change of the electron distribution by reduction of a nitro substituent, the flexible monoether structure is even more likely. The fact that covalent labeling occurs in both α and β, strongly supports rotational mobility of the phenyl ring that carries the reactive group, and moreover supports a close proximity of a certain part of α subunit to the catalytic nucleotide binding site on β subunit [6].

That binding of the ADP analogues in fact occurs to a catalytic site, is substantiated by the competitive effect on phosphorylation [10] and by the protective effect of substrates on covalent binding (Table V). Protection is strongest when both, ADP and phosphate are present. The fact that phosphate alone attenuates covalent binding, suggests interference of the nucleotide analogues with the phosphate binding site as well. Actually screening of the phosphate binding site by the trinitrophenyl residue was concluded to be an important reason for inhibition of photophosphorylation by TNP-ADP [10]. The effect of phosphate on covalent labeling which is stronger in α than in β subunit (Table V), may indicate that the phosphate binding site is actually located in α subunit. Although photolabeling with 4-azido-2-nitrophenyl phosphate used as a phosphate analogue, yielded predominant incorporation into β subunit of CF$_2$ [41], this result does not really prove the localization of the phosphate binding site in β. As in the employed compound the photoreactive azido group is in para-position to the phosphate residue, the phosphate binding domain may well be in α while covalent labeling occurs to a juxtaposed peptide strand of β subunit. For the elucidation of the enzymatic mechanism, the precise localization of the phosphate binding site would be highly significant.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (grant Str 103/17-1 and Sonderforschungsbereich 189) and by Fonds der Chemischen Industrie. The authors thank Dr. Susanne Bickel-Sandkötter and Dr. Jürgen Schumann for critically reading and Mrs. Rita Reidegeld for typing the manuscript.