THE INTERACTION OF ENERGY TRANSFER INHIBITORS WITH THE ADENINE NUCLEOTIDE BINDING SITES ON SOLUBLE CHLOROPLAST COUPLING FACTOR 1

Varda SHOSHAN[+] and Bruce R. SELMAN*

Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, WI 53706, USA

Received 21 August 1979

1. Introduction

Coupling factor 1 (CF_1), isolated from chloroplasts, is believed to be the protein which catalyzes the synthesis of ATP in photophosphorylation [1]. The latent, isolated protein exhibits ATPase activity when properly activated by heat [2], trypsin [3], or dithiothreitol [4].

It has been shown that the protein contains multiple nucleotide binding sites [5–11]. However, the relationship between the 'tight' nucleotide binding sites ($K_d \sim \mu M$) in the isolated, latent or activated protein and the ATPase catalytic site(s) is unclear. It has been argued that the rate of association of nucleotides such as ϵADP, ϵAMP-P(NH)P [5] or formycin di- and triphosphate [11] with the tight sites of the latent enzyme is too slow for such sites to be involved in catalytic turnover. However, it was reported [11] that the association of nucleotides with the activated enzyme is much faster and suggested that this change in association kinetics may reflect the activation of the ATPase. The binding site for the ATP analogue AMP-P(NH)P, exposed upon heat activation of CF_1, has been suggested as the active site for the ATPase, whereas the other tight binding site(s) are regulatory [5].

Here we report on the interaction of energy transfer inhibitors with the adenine nucleotide binding sites. Some energy transfer inhibitors, e.g., phlorizin, tentoxin and quercetin, are found to stimulate the binding of adenine nucleotides to CF_1 by exposing additional binding site(s) on the protein. On the other hand, dicyclohexylcarbodiimide (DCCD) inhibits the ATPase activity (of activated CF_1) and eliminates one of the nucleotide binding sites which is exposed by the activation of the latent CF_1-ATPase or in the presence of tentoxin. These results suggest that the binding site for adenine nucleotides, exposed by the activation of CF_1 or the presence of energy transfer inhibitors, may be the active site for ATP hydrolysis.

2. Materials and methods

Chloroform-released CF_1 was isolated and purified as in [11,14]. The protein was stored as a suspension in 50% saturated ammonium sulfate containing 1 mM EDTA, 4 mM ATP, and 20 mM tricine–NaOH (pH 7.1). Prior to use, ammonium sulfate and readily exchangeable nucleotides were removed by chromatography on Sephadex G-50 (1.0 × 10 cm) equilibrated with 1 mM EDTA and 40 mM tricine–NaOH (pH 8.0). The ATP, ADP, and AMP content of the latent and DTT-activated proteins was examined by the methods in [15]. The enzyme, after gel filtration, contained about 1 mol ADP/mol CF_1, 0.16 mol ATP/mol CF_1 and no detectable AMP. Disc-gel electrophoresis demonstrated that the protein was ⩾ 97% pure. Pro-

Abbreviations: CF_1, chloroplast coupling factor 1; AdN, adenine nucleotide; tricine, *N*-[Tris(hydroxymethyl)methyl] glycine; AMP-P(NH)P, adenylyl imidodiphosphate; DCCD, dicyclohexylcarbodiimide; EDTA, ethylenediamine tetraacetic acid

[+] Present address: Banting and Best Department of Medical Research, Charles H. Best Institute, University of Toronto, Toronto, Ontario M5G 1L6, Canada

* To whom all correspondence should be addressed

tein concentration was determined as in [16] with bovine serum albumin as a standard and assuming mol. wt 325 000 for CF_1 [17]. The Ca^{2+}-ATPase activity of the protein was determined as in [14] after heat [2] or dithiothreitol [4] activation.

Radioactive nucleotides, [2,8-^{3}H]ADP and [8-^{3}H]-AMP-P(NH)P, were purchased from New England Nuclear and [2,8-^{3}H] from ICN. [γ-^{32}P]ATP was prepared from ADP and [^{32}P]phosphate by photophosphorylation and was purified as in [18].

The binding of labeled adenine nucleotides to CF_1 (0.2 mg/ml) was measured by the chromatography–centrifugation procedure in [19] except that the Sephadex G-50 fine was equilibrated in 20 mM tricine–NaOH (pH 8.0). Incubation mixtures for the binding assay contained in 0.22 ml total vol., 50 mM tricine–NaOH (pH 8.0), 25 mM NaCl and 10 μM labeled nucleotide (at ~3 × 10^5 cpm/nmol), and other reagents as indicated in the legends. After incubation at 37°C for 30 min, 0.1 ml sample was loaded onto a 1 ml packed Sephadex G-50 column and centrifuged for 2 min at ~1200 × g. Scintillation cocktail [12] 5.0 ml, was added to the column effluent and the radioactivity was measured in a Beckman LS100 liquid scintillation counter.

3. Results

The effects of tentoxin, phlorizin, and quercetin on the binding of ADP or ATP to CF_1 are shown in table 1. Under the conditions used (the absence of divalent cations), all of the inhibitors increase the total amount of adenine nucleotide bound to CF_1 2–3-fold. Similar results have been reported for tentoxin [12,13].

Table 1
The effect of several energy transfer inhibitors on ADP and ATP binding to isolated, latent CF_1

Inhibitor	mol AdN bound/CF_1	
	ADP	ATP
None	0.37	0.63
Phlorizin (2.5 mM)	0.63	1.01
Phlorizin (10 mM)	0.94	n.d.
Tentoxin (20 μM)	0.65	1.25
Tentoxin (200 μM)	1.15	1.36
Quercetin (50 μM)	0.52	1.02
Quercetin (200 μM)	0.62	0.96

n.d. = not determined

Adenine nucleotide binding was measured as in section 2

Figure 1 shows the correlation between the phlorizin stimulation of ATP binding to the soluble coupling factor and the inhibition of the ATPase activity. A linear relationship between the increase in the total bound nucleotide and the inhibition of the ATPase activity is obtained (fig.1, inset).

Heat activation of the latent CF_1 has been the cause reported [5] for the appearance of an additional binding site for the ATP analogue AMP-P(NH)P. This observation has been confirmed for the DTT-activated CF_1 (table 2). The effect, however, is most significant in the absence of divalent cations (0.55 compared to 2.38 AMP-P(NH)P/CF_1). Table 2 also shows that DTT-activation of CF_1 increases the binding of not only AMP-P(NH)P, but also of ADP and ATP.

The relationship between the site(s) exposed by DTT-activation of CF_1 and the site(s) exposed in the

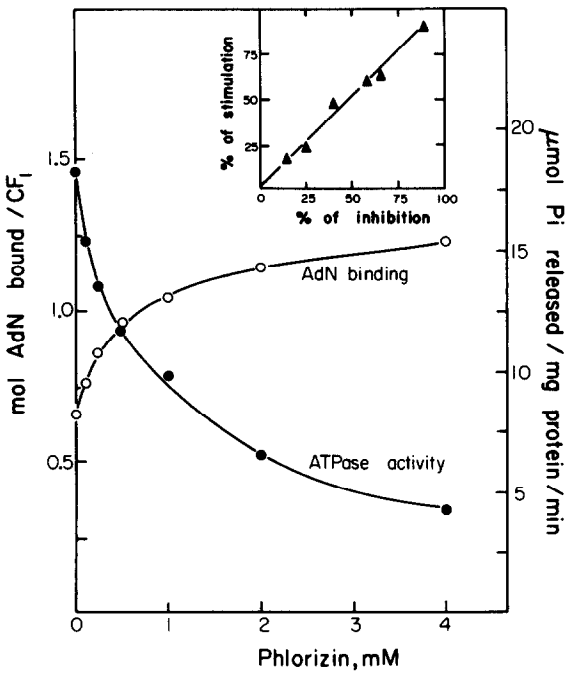


Fig.1. Correlation between the phlorizin inhibition of the ATPase activity and stimulation of ATP binding. Conditions for [^{3}H]ATP binding or ATPase activity of the heat activated enzyme were as in section 2.

Table 2
Comparison between the effects of DTT-activation, Mg^{2+} and phlorizin on the binding of different labeled nucleotides

Nucleotide	mol AdN bound/CF_1					
	Latent CF_1			DTT-activated CF_1		
Additions:	None	Mg^{2+}	Phlorizin	None	Mg^{2+}	Phlorizin
[^{3}H]ADP	0.50	1.39	0.89	0.99	1.18	1.44
[^{3}H]ATP	0.89	1.43	1.51	2.15	2.07	2.30
[^{3}H]AMP-P(NH)P	0.55	1.76	0.61	2.38	2.34	2.29
[γ-^{32}P]ATP	0.52	0.99	0.95	1.18	0.96	1.14

CF_1 was activated by incubation for 30 min at 37°C with 50 mM DTT (pH 8.0). DTT was removed by the centrifugation-chromatography procedure [18]. Conditions for nucleotide binding were as in section 2 except that the reaction mixtures contained 5 mM phlorizin or 2 mM $MgCl_2$ where indicated

presence of an energy-transfer inhibitor, phlorizin, is also shown in table 2. Both Mg^{2+} and phlorizin increase the binding of all of the nucleotides to the latent enzyme but have only a marginal effect on the binding to the DTT-activated CF_1. A combination of both Mg^{2+} and phlorizin does not further increase the binding to the latent enzyme (data not shown). These results suggest that the nucleotide binding site(s) exposed by Mg^{2+} or energy transfer inhibitors is the same site which is exposed by the activation of CF_1 to an active ATPase. However, the effect of DTT-activation on nucleotide binding persists even after removal of DTT, whereas the stimulatory effect of energy transfer inhibitors or Mg^{2+} requires their continual presence during the binding assays (data not shown).

The number of ADP binding site(s) and the dissociation constants obtained with latent CF_1 under different conditions are summarized in table 3. The latent enzyme has a single, strong binding site with $K_d \sim 0.7$ μM. Under similar conditions, a single binding site per CF_1 was also found [10,20]. However, in the presence of either Mg^{2+}, tentoxin, or phlorizin the number of the ADP binding sites increases to 3 and the affinity of the enzyme for ADP decreases. DTT activation of CF_1 increases the number of ADP binding sites to 2 or 3. (V.S., B.R.S., submitted). The apparent increase in the number of ADP binding sites by the above compounds or by the activation of the enzyme might be due to an acceleration of ADP incorporation into CF_1 and/or to a stabilization of the pre-bound nucleotides to loose binding sites and which are easily dissociated from the enzyme during the binding assay (Sephadex gel chromatography).

We have shown (V.S., B.R.S., submitted) that, under certain conditions, DCCD interacts with soluble CF_1 to inhibit the ATPase activity. Figure 2 shows that DCCD inactivation of the CF_1-ATPase also results in the inhibition of ADP binding to the DTT-activated enzyme. Maximal inhibition of the ATPase activity is accompanied by the elimination of one ADP binding site. A linear relationship between the loss of ATPase activity and the disappearance of the ADP binding site is observed (fig.2, inset). Table 4 shows that DCCD-modification of CF_1 inhibits not only the ADP binding to the DTT-activated CF_1, but

Table 3
Dissociation constants (K_d) and numbers of binding sites (n) for the binding of ADP to CF_1

Additions	n	K_d (μM)
None	1	0.7
$MgCl_2$ (2 mM)	3	1.9
Tentoxin (350 μM)	3	1.5
Phlorizin (9 mM)	3	2.6

Latent CF_1 was incubated for 30 min at 37°C with varying concentrations of [^{3}H]ADP. Incubation mixtures contained 50 mM tricine–NaOH (pH 8.0) and 2.5 mM NaCl. K_d and n were calculated from Scatchard plots

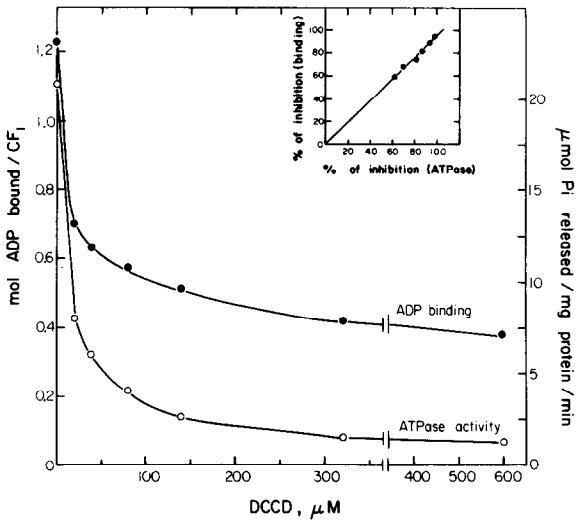


Fig.2. Correlation between the DCCD inhibition of the ATPase activity and ADP binding. DTT-activated CF_1 was obtained as in table 2. The activated enzyme was incubated for 30 min at 37°C with the indicated concentrations of DCCD in 100 mM MOPS buffer (pH 7.0). Aliquots of 5 μl and 50 μl were assayed for ATPase activity and ADP binding, respectively. The % inhibition of ADP binding by DCCD (inset) was calculated for the values after subtraction of the DCCD-insensitive portion of the binding (0.35 ADP bound/F_1).

also the tentoxin stimulation of ADP binding to the latent CF_1. DCCD only slightly affects the binding of ADP to the latent CF_1.

4. Discussion

It is apparent that the interaction of soluble CF_1 with the energy transfer inhibitors, tentoxin, quercetin, and phlorizin increases the binding of adenine nucleotides to the enzyme in a manner similar to DTT-activation of the ATPase or heat activation [5]. The question that arises, of course, is how the adenine nucleotide binding site(s) exposed by these treatments relates to:

(i) The active site of the ATPase and/or

(ii) The so-called 'tight' binding site of membrane-bound CF_1 whose nucleotides can be exchanged upon energization of the thylakoid membrane (see [21]).

We have suggested that the tightly bound ADP occupies either the active or a regulatory site for ATPase activity [22].

We have reported [12,13] that tentoxin induces an exchange of tightly bound ADP for medium ADP with the soluble protein. If this adenine nucleotide binding site is filled with a radioactively labeled adenine nucleotide and the CF_1 reconstituted with partially resolved thylakoid membranes, the bound adenine nucleotide exchanges with medium nucleotides upon energization of the membrane. Therefore, we have argued that the 'tight' binding site exposed either by tentoxin (with the soluble coupling factor) or light (with the membrane-bound protein) are identical [12,13]. This, however, is probably not the only change in the properties of the binding sites

Table 4
Effect of DCCD modification of CF_1 on ADP binding to the latent and activated proteins

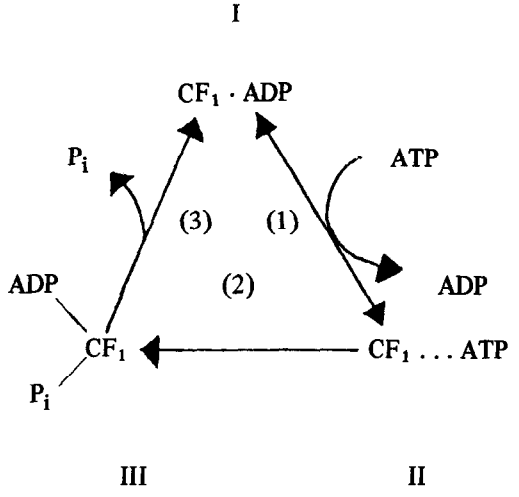
CF_1 preparation	mol ADP bound/CF_1		ATPase activity (μmol P_i released/mg protein/min)
	– Tentoxin	+ Tentoxin	
Latent	0.89	2.08	2.09
DCCD-modified, latent	0.65	1.15	0.1
DTT-activated	1.78	2.13	13.8
DCCD-modified, DTT-activated	0.76	1.40	1.0

DCCD-modified CF_1 was obtained as in fig.2, and DTT-activated CF_1 as in table 2. ATPase activity and ADP binding were measured as in section 2. Tentoxin was 200 μM

induced upon activation of the coupling factor.

An analysis of table 2 shows that, although energy transfer inhibitors induce adenine nucleotide binding to CF_1, they are not as effective as DTT-activation. The binding of adenine nucleotides to DTT-activated CF_1 is not influenced by energy transfer inhibitors (except DCCD, see discussion below). This result suggests that the binding sites exposed by DTT activation of CF_1 encompass the binding sites exposed by energy transfer inhibitors.

There are quantitative differences in the absolute level of binding depending on the nature of the nucleotide, i.e., ATP or AMP-P(NH)P versus ADP and even [^{3}H]ATP versus [γ-^{32}P]ATP (table 3). In order to explain these differences, we have suggested the following model.



State I represents a tight complex between CF_1 and ADP and is the stable form of the isolated, purified CF_1 [11,23–25]. The tightly bound ADP is exchanged with ATP in the medium under certain conditions [12,13,20] to form state II. The ATP in the CF_1–ATP complex is rapidly hydrolyzed to form bound P_i and ADP (state III) (table 2). The increase in nucleotide binding by the activation of CF_1 could be due to an acceleration of reaction (1) or, in the case of energy transfer inhibitors, inhibition of reaction (2). This suggestion is supported by the observation that, upon activation of CF_1, a marked increase in the rate of nucleotide binding is observed [11]. Accordingly, we find that when the DTT-activated enzyme is incubated with [^{3}H]ATP or [^{3}H]AMP-P(NH)P, $\geqslant 2$ binding sites are filled. We suggest that one of these is the ATPase active site (AMP-P(NH)P is a strong competitive inhibitor of the ATPase [5]) and the other sites are binding sites that may have a regulatory function. On the other hand, incubation of the activated enzyme with [^{3}H]ADP or [γ-^{32}P]ATP reveals 1 less site. We suggest that the binding of [^{3}H]ADP under these conditions is not to the active site, but rather to a regulatory site. ADP has been shown [26] to be an allosteric inhibitor of the ATPase which would suggest binding to a site other than the active site. [γ-^{32}P]ATP binds to both types of sites, but because the ATPase is active, the bound $^{32}P_i$ is lost and the binding to the active site is not seen. Thus, the binding induced by energy transfer inhibitors and activation of the ATPase is a mixture of exchange induced both at the active site and regulatory site(s).

Treatment of the protein with DCCD causes a concommitant loss of the activity of the enzyme and one adenine nucleotide binding site. It is tempting to speculate that the loss of activity by DCCD modification of CF_1 is due to an inhibition of nucleotide exchange at the tight-binding site which has been suggested to be the active site. However, it is not yet clear whether the lost binding site is the ATPase active site or a regulatory site. This is particularly important because chemical modification of only the α-subunit of F_1-ATPase causes a full inactivation of the enzyme [27], whereas the active site is thought to be on the β-subunit [28]. Clearly, there is an interaction between the 2 types of binding sites. The effect of DCCD treatment on the activities of the F_1-ATPase has been reported [29] and for CF_1 has been demonstrated and detailed (V.S., B.R.S., submitted). DCCD binds covalently to the β-subunit of CF_1 and the inhibition of the hydrolytic activity parallels the loss of 1 nucleotide binding site. These findings support the location of the active site for ATPase activity on the β-subunit of CF_1 [28]. DCCD modification of CF_1 can be used to provide important insights into the role and function of 1 adenine nucleotide binding site of CF_1, both in the soluble and membrane-bound CF_1.

Acknowledgements

This research was supported in part by grants from the College of Agricultural and Life Sciences, University of Wisconsin-Madison, a Harry and Evelyn Steenbock Career Advancement Award in Biochemistry, grant PCM7911025 from the National Science Foundation, and the United States Department of Agriculture/SEA (5901-0410-8-0071-0) from the Competitive Research Grants Office.

References

[1] Avron, M. (1963) Biochim. Biophys. Acta 77, 699–702.
[2] Farron, F. and Racker, E. (1970) Biochemistry 9, 3829–3936.
[3] Vambutas, V. K. and Racker, E. (1965) J. Biol. Chem. 240, 2660–2667.
[4] McCarty, R. E. and Racker, E. (1968) J. Biol. Chem. 247, 6506–6510.
[5] Cantley, L. C., jr. and Hammes, G. G. (1975) Biochemistry 14, 2968–2975.
[6] Girault, G., Galmiche, J. M., Michel-Villaz, M. and Thiery, J. (1973) Eur. J. Biochem. 38, 1173–1178.
[7] Vandemeuln, D. L. and Govindjee (1977) Eur. J. Biochem. 78, 585–589.
[8] Tiefert, M. A., Roy, H. and Moudrianakis, E. N. (1977) Biochemistry 16, 2396–2403.
[9] Girault, G. and Galmiche, J. M. (1977) Eur. J. Biochem. 77, 501–510.
[10] Banai, M., Shavit, N. and Chipman, D. M. (1978) Biochim. Biophys. Acta 504, 100–107.
[11] Shoshan, S., Shavit, N. and Chipman, D. M. (1978) Biochim. Biophys. Acta 504, 108–122.
[12] Reimer, S. and Selman, B. R. (1979) Biochim. Biophys. Acta 545, 415–423.
[13] Selman, B. R. and Selman-Reimer, S. (1979) FEBS Lett. 97, 301–304.
[14] Lien, S. and Racker, S. (1971) Methods Enzymol. 23, 547–556.
[15] Kimmich, G. A., Randles, J. and Brand, J. S. (1975) Anal. Biochem. 69, 187–206.
[16] Bradford, M. M. (1976) Anal. Biochem. 72, 248–254.
[17] Farron, F. (1970) Biochemistry 9, 3823–3828.
[18] Magnussen, R. P., Portis, A. R., jr and McCarty, R. E. (1976) Anal. Biochem. 72, 653–657.
[19] Penefsky, H. S. (1977) J. Biol. Chem. 2891–2899.
[20] Carlier, M. and Hammes, G. G. (1979) Biochemistry in press.
[21] Harris, D. A. (1978) Biochim. Biophys. Acta 463, 245–273.
[22] Shoshan, V. and Selman, B. R. (1979) J. Biol. Chem. 254, in press.
[23] Magnussen, R. P. and McCarty, R. E. (1976) Biochem. Biophys. Res. Commun. 80, 1283–1289.
[24] Harris, D. A. and Slater, E. C. (1975) Biochim. Biophys. Acta 387, 335–348.
[25] Strotmann, H. and Bickel-Sandkötter, S. (1977) Biochim. Biophys. Acta 460, 126–135.
[26] Nelson, N. (1977) Biochim. Biophys. Acta 456, 314–338.
[27] Lunardi, J. and Vignais, P. V. (1979) FEBS Lett. 102, 23–28.
[28] Deters, D. W., Racker, E., Nelson, N. and Nelson, H. (1975) J. Biol. Chem. 250, 1041–1047.
[29] Pougeois, R., Satre, M. and Vignais, P. V. (1979) Biochemistry 18, 1408–1412.