Biochimica et Biophysica Acta 894 (1987) 277–283
Elsevier

BBA 42657

Dissociation-reconstitution experiments support the presence of two catalytic β-subunits in mitochondrial F_1

P. Nieboer, A.F. Hartog and J.A. Berden

Laboratory of Biochemistry, University of Amsterdam, Amsterdam (The Netherlands)

(Received 2 July 1987)

Key words: ATPase, F_1-; Quaternary structure; Chemical modification; β-Subunit; Catalytic site; (Bovine heart mitochondria)

Mitochondrial F_1, inactivated to various extents with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), was dissociated with LiCl and reconstituted after removal of the salt. This procedure resulted in a reactivation that corresponded with a reactivation theoretically expected on the basis of the assumption that the reassociation of β-subunits into native F_1 molecules is random and that two out of the three β-subunits are directly involved in catalysis. Repeated inactivation of such reactivated F_1, followed by the same dissociation-association procedure, resulted in similar data. After inactivation of F_1 by covalent binding of 2-N-AT(D)P to one catalytic site, no reactivation upon dissociation-reassociation was obtained due to the fact that such modified F_1 did not dissociate under the experimental conditions used.

Introduction

After the subunit composition of mitochondrial F_1 had been established [1,2], the discussion on the number of catalytic sites continued. These catalytic sites were thought to be located on the β-subunits [3]. The arguments in favour of the presence of three cooperative catalytic sites, i.e., three β-subunits, three tightly bound nucleotides [4] supposed to be located on non-catalytic α-subunits, and three rapidly exchangeable sites [5], were supported by the kinetic analysis presented by Gresser et al. [6], and by the inactivation of F_1 with 3 mol of *p*-fluorosulphonylbenzoyl-5′-adenosine per mol of F_1 [7]. On the other hand, the detailed analysis of the photolabelling data by our group led to the conclusion that F_1 contains two catalytic sites on β-subunits, two non-catalytic exchangeable sites located at α–β interfaces, and two non-exchangeable sites located on the third α–β couple, one on the third β-subunit and the other one the third α–β interface [8,9]. These latter conclusions were in agreement with the data on binding of dicyclohexylcarbodiimide (DCCD) [10] and the structural data on F_1 [11]. The recent finding of Kironde and Cross [12] that one non-catalytic site (previously considered to be non-exchangeable [5]) is in fact exchangeable further supports the two-site model. The kinetic data can also be explained well with such a model [13].

Recent papers by Wang [14–16] present data that support the proposal of only one catalytic site. Since such a proposal seems contradictory to many well-documented phenomena, such as the cooperativity between catalytic sites [8,17], we performed experiments similar to those of Wang. The conclusion is reached that these experiments con-

Abbreviations: NBD-Cl, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (also named 4-chloro-7-nitrobenzofurazan); F_1, mitochondrial ATPase; DCCD, dicyclohexylcarbodiimide.

Correspondence: J.A. Berden, BCP Jansen Instituut, Plantage Muidergracht 12, 1018 TV Amsterdam, The Netherlands.

firm the presence of two cooperative catalytic β-subunits in F_1.

Materials and Methods

Bovine heart mitochondrial F_1-ATPase was isolated according to the method described by Knowles and Penefsky [18] and stored in liquid nitrogen in a medium containing 10 mM Tris-HCl (pH 7.5), 4 mM ATP, 250 mM sucrose and 4 mM EDTA. Prior to use, F_1 was precipitated with $(NH_4)_2SO_4$ and dissolved in buffer A. (Buffer A contained 50 mM Hepes-NaOH (pH 7.0), 2 mM EDTA, 5 mM ATP and 25% (v/v) glycerol [16]). $(NH_4)_2SO_4$ was removed by means of a centrifuge column packed with Sephadex G-50 and equilibrated with buffer A. Protein concentrations were determined with the BioRad protein assay. The starting concentration of F_1 was about 6 mg/ml with a specific activity of 140 μmol/min per mg.

Pyruvate kinase (in glycerol), lactate dehydrogenase (in glycerol), Hepes, NADH, ATP and phospho*enol*pyruvate were obtained from Boehringer Mannheim. NBD-Cl was produced by Serva, dithiothreitol by Sigma, and Sephadex G-50-Coarse by Pharmacia. 2-Azido-ATP was synthesized as described previously [9].

ATP-hydrolysis assay. The assay medium contained 33 mM Tris-HCl (pH 8.0), 83 mM sucrose, 10 mM $KHCO_3$, 6 mM $MgCl_2$, 5 mM ATP, 0.5 mM phospho*enol*pyruvate, 250 μM NADH, 2.5 U pyruvate kinase/ml and 2 U lactate dehydrogenase/ml. The oxidation of NADH at 30 °C was followed on a Zeiss M4QIII spectrophotometer at 340 nm.

Preparation of the centrifuge columns. The centrifuge columns (5 × 1 cm) consisting of Sephadex G-50-Coarse equilibrated with buffer A were pre-centrifuged for 1 min at 2000 r.p.m. in a Homef LC-30 table centrifuge using a volume of buffer A of 100 μl. The columns used for the F_1 treated with LiCl were chilled on ice. 100 μl of each protein sample was centrifuged at a speed of about 2400 r.p.m. for 1 min. The columns consisting of Sephadex-G-50-Coarse equilibrated with buffer B (see labelling with 2-azido-ATP) were spun and pre-centrifuged at 2000 r.p.m. for 1 min using the same centrifuge.

Labelling with NBD-Cl. Labelling with NBD-Cl was performed in the dark at 20 °C in buffer A containing 75 μM of NBD-Cl (stock solution 2 mM in ethanol) [19]. Inhibition of hydrolysis activity was calculated from the activity values obtained with and without 125 μM dithiothreitol. The value obtained in the presence of dithiothreitol was considered to be the corresponding 100% value.

Labelling with 2-azido-ATP. Labelling with 2-azido-ATP was performed in a buffer containing 50 mM Hepes-KOH (pH 7.5), 150 mM sucrose and 4 mM $MgCl_2$ (buffer B). After precipitation with $(NH_4)_2SO_4$, F_1 was dissolved in buffer B and $(NH_4)_2SO_4$ was removed by means of a centrifuge column consisting of Sephadex G-50-Coarse equilibrated with buffer B. After this, 2-azido-ATP (in methanol) was added to a concentration of 1 mM. 1 min later $(NH_4)_2SO_4$ precipitation followed, and again the $(NH_4)_2SO_4$ was removed by means of a centrifuge column of the same type as mentioned above. The F_1 was now photolabelled by making use of a specially designed xenon-flash unit (100 J per flash; flash duration, ≈ 50 μs). Between the F_1 samples and the flash tube, a 1 mm thick piece of glass was put in order to cut off most of the light below 300 nm and prevent protein damage. The first two irradiation sessions were performed at a distance of 3 cm from the flash tube in a shallow vessel made of aluminium foil with an interval of 30 min. After each irradiation session, part of the F_1 sample was removed. 30 min later, these samples were irradiated at a distance of 1.5 cm from the flash tube and this procedure was repeated twice with intervals of 30 min. Here also part of the F_1 sample was removed after each irradiation session. Each irradiation session consisted of 10 flashes fired with intervals of 15 s. After irradiation, the F_1 samples were put in buffer A by means of a centrifuge column equilibrated with buffer A. The same labelling and irradiation procedure was done with F_1 samples incubated with ATP instead of 2-azido-ATP. The hydrolysis activity of these samples was taken as 100%. After the procedure described above, all samples were dissociated with LiCl and after reconstitution the values of the ATP samples were again taken as 100%.

Dissociation and reconstitution. Samples of F_1 with different degrees of inhibition of ATP hy-

drolysis were chilled on ice for 3 min and then mixed at a ratio of 1 : 1 with ice-cold buffer A containing 6 M LiCl. After mixing for 100 s, the LiCl was removed by means of a centrifuge column. Reconstitution was optimal 15 min after removal of LiCl and stayed constant for at least 1 h. Inhibition of hydrolysis was determined again and the samples were relabeled until nearly total inhibition occurred, after which the LiCl treatment was repeated. The method described above is the method used by Wang et al. [16], except that we used an incubation time of 100 s instead of 3.5 min and repeated the LiCl treatment.

Polyacrylamide gel electrophoresis. In order to determine the level of dissociation of F_1 samples labelled with 2-azido-ATP, a non-denaturating polyacrylamide gel was prepared. The method used was that described by Knowles and Penefsky [18], except that ATP and EDTA were omitted and instead of tube gels slab gels were made. F_1 samples with different degrees of inhibition were treated with LiCl for 100 s, as described above. After LiCl treatment the samples were transferred to a buffer containing 0.25 M sucrose and 50 mM Hepes-KOH (pH 7.5) by means of a centrifuge column and directly placed on the gel. Staining was done with Coomassie brilliant blue.

Statistics. In order to evaluate the results of the experiments, some statistical calculations were made. The degree of inhibition was considered to be the same as the amount of label on the F_1 [19,22]. The assumptions made were:
(i) labelled and unlabelled β-subunits behave identically upon treatment with LiCl;
(ii) at reconstitution the β-subunits integrate randomly into F_1;
(iii) labelling with NBD-Cl takes place at one catalytic β-subunit.

If we consider the three β-subunits of F_1 to have different functions in the enzyme, we can call them β-1, β-2 and β-3. The β-1 subunit in this calculation is the subunit where the label is incorporated in the enzyme. The total amount of label on the enzyme is called x (mol of label/mol of F_1). Inactivation after reconstitution is expressed by r. At total inhibition $r = 1$ and at zero inhibition $r = 0$. Before LiCl treatment there are only two kinds of F_1 possible:
(i) β-1, β-2, β-3 = X, 0, 0 (X = labelled, 0 = unlabelled); and
(ii) β-1, β-2, β-3 = 0, 0, 0.
After reconstitution many more kinds of F_1 are possible (see Table I).

The amount of labelled β-subunits is $\frac{1}{3}x$ and the amount of unlabelled β-subunits is $1 - \frac{1}{3}x$. The expected values for r were calculated for three kinetic models:
(i) One catalytic site:

$$r = \tfrac{1}{3}x$$

(ii) Two cooperative catalytic sites:

$$r = 2(1-\tfrac{1}{3}x)^2 \cdot \tfrac{1}{3}x + 3(1-\tfrac{1}{3}x)(\tfrac{1}{3}x)^2 + (\tfrac{1}{3}x)^3$$

(iii) Three cooperative catalytic sites:

$$r = 3(1-\tfrac{1}{3}x)^2 \cdot \tfrac{1}{3}x + 3(1-\tfrac{1}{3}x)(\tfrac{1}{3}x)^2 + (\tfrac{1}{3}x)^3$$

Results and Discussion

Previously [20] we have used the dissociation of β-subunits from F_1 in the presence of LiCl to develop a simple procedure for the determination of the F_1 content of submitochondrial particles. Isolated F_1 was also dissociated with 0.85 M LiCl at pH 6.0, but appreciable reconstitution was only obtained when LiCl was removed before the inactivation was completed [21]. This method for

TABLE I

$x = 0$			$x = 1$			$x = 2$			$x = 3$		
β-1	β-2	β-3	β-1	β-2	β-3	β-1	β-2	β-3	β-1	β-2	β-3
0	0	0	X	0	0	X	X	0	X	X	X
			0	X	0	0	X	X			
			0	0	X	X	0	X			

dissociation-reconstitution was recently improved by Wang [15], using higher concentrations of LiCl in the presence of glycerol and ATP. To obtain reproducible results, both at the level of reconstitution of specific activity and at the level of the recovery of protein we modified the procedure at the column centrifugation step. Also the time of incubation influenced the final reconsituted activity. In Fig. 1 it can be seen that inactivation was reached within 30 s and that after 100 s the reconstitued activity was still 60–70% of the original activity. After longer incubation times the reconstituted activity decreased. Binding of NBD-Cl to F_1 was followed both spectrophotometrically and by measuring the activity. For the experiment shown in Fig. 2 samples were taken at various levels of binding (inactivation). On the basis of earlier data [19,22] it was assumed that inactivation is proportional with the binding of 1 mol of NBD-Cl/mol of F_1.

After dissociation followed by reconstitution, the sample that was originally inactivated to the largest extent, was again treated with NBD-Cl. The time-course of the binding (inactivation) was identical with the time-course of the original binding, indicating that inactivation was again due to binding of NBD-Cl to one catalytic site. Fig. 3 shows the results of the same experiment when the activities after reconstitution were measured. As a control for full activity, both unmodified F_1 and

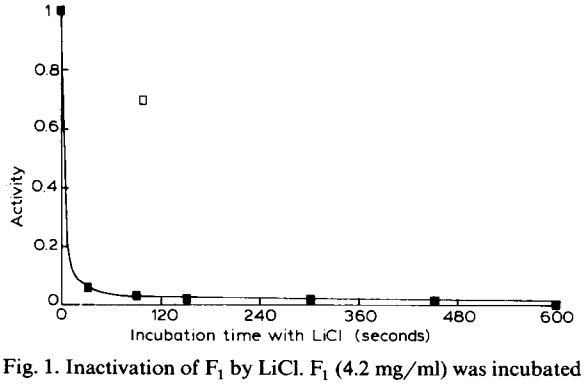

Fig. 1. Inactivation of F_1 by LiCl. F_1 (4.2 mg/ml) was incubated at 20 °C in medium A and at $t = 0$ an equal volume of cold medium A containing 6 M LiCl was added. At the indicated times, samples were withdrawn for measurement of the ATPase activity (■). After 100 s a sample was desalted on a column as described in Materials and Methods and the activity measured after 15 min (□).

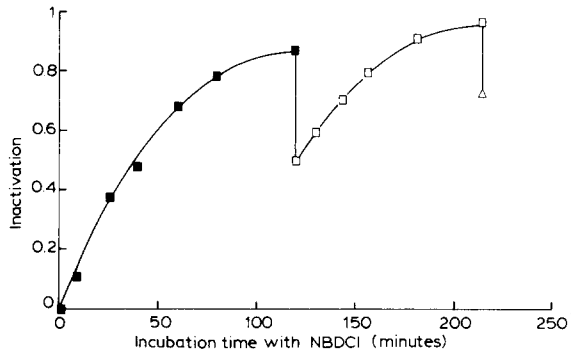

Fig. 2. Inactivation of F_1 with NBD-Cl. F_1 (6 mg/ml) was incubated at 20 °C in the dark with 75 μM NBD-Cl in medium A. After the indicated time intervals, samples were withdrawn for measurement of ATPase activity (■) and treatment with LiCl. The sample taken after 120 min was incubated again (after the dissociation-reconstitution procedure) with 75 μM NBD-Cl (□) and after renewed treatment with LiCl and reactivation was determined of the sample with the highest inactivation (△). In both incubations the $t_{1/2}$ values for the binding of NBD-Cl were identical (40 min).

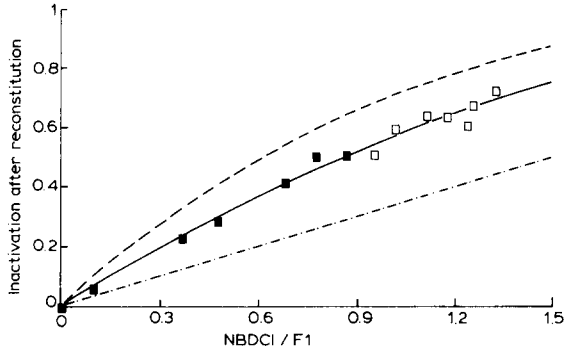

Fig. 3. Reactivation of NBD-modified F_1. All samples taken from the incubation described in Fig. 2 were treated with LiCl and desalted as described in Materials and Methods, and the ATPase activities after reconstitution were measured. The activity in the presence of dithiothreitol was taken as the 100% value. The amount of bound NBD was assumed to be proportional with the inactivation before LiCl treatment, 100% inactivation being equivalent with 1 mol of NBD bound per mol of F_1 (■). All samples were incubated again with NBD-Cl after the first dissociation-reconstitution procedure, and the additional amount of bound NBD was also assumed to be proportional to the additional inactivation, since only F_1 with no modified catalytic β-subunit can bind NBD-Cl (□). The curves ------, ———, and –·–·– are theoretical curves calculated on the basis of random incorporation of the dissociated β-subunits into F_1 and the involvement of three, two or one β-subunit, respectively, in the catalytic process of ATP hydrolysis.

the activities after treatment with dithiothreitol were used, giving identical values within 2%. The open symbols in the figure represent the data obtained when the samples after the first dissociation-reconstitution step were treated again with NBD-Cl till nearby full inhibition again and then subjected to the dissociation-reconstitution procedure for a second time. It is clear that all points are in agreement with the theoretical curve for random incorporation of β-subunits into F_1 with two of the three β-subunits being involved in cooperative catalysis. The data of Wang et al. in Ref. 16 also fit with this curve. In Ref. 15 a higher level of reactivation was reported, suggesting that only one of the β-subunits in F_1 was catalytically active. In Table I of that reference it can be seen, however, that a high ratio of the specific activity of labelled F_1-specific activity of dithiothreitol-treated F_1 was only obtained after long incubation times, under which conditions reconstituted activity measured after dithiothreitol-treatment was substantially lower than after shorter incubation times, while the activity before the dithiothreitol-treatment is much less affected by the length of the incubation period. Assuming that the migration of bound NBD to the lysine residue is not higher than estimated by Wang, the result can be explained by the assumption that after long incubation times the NMB-modified β-subunits are less reconstitutively active than the unmodified subunits. The control with unmodified F_1 is missing in this experiment. Also the data in Ref. 14 are not conclusive. The data can very well be explained by the (reasonable) assumption that when 1 mol of NBD per mol of F_1 is bound to the tyrosine of a catalytic β-subunit, the second catalytic subunit is fully protected against NBD-Cl and that for the very slow additional binding the non-catalytic subunit is responsible, together with the migration of bound NBD to a lysine residue, both in the modified catalytic subunit and the non-catalytic subunit.

Seemingly, our results can be made compatible with a model for three catalytic β-subunits if it is assumed that NBD-modified β-subunits are less reconstitutively active than unmodified subunits, resulting in a higher (relative) reactivation than predicted for this model. Such an explanation, however, is ruled out by the fact that the reconstituted activity (after treatment with dithiothreitol) obtained with modified F_1 is exactly the same as that obtained with unmodified F_1, indicative for equal constitutive activity of modified and unmodified β-subunits.

Another way to make the results compatible with a model for three catalytic sites is to suppose a loss of NBD during the dissociation-association procedure. Both our own spectral analysis and the data of Wang [14–16] contradict this possibility. To fit the data with a 3 site-model about 25% of the bound NBD should have been lost, too much to escape detection. Also migration of NBD to a lysine residue results in a higher than theoretical reactivation relative to the activity after treatment with dithiothreitol. But in that case the reconstituted activity of modified F_1 should be lower than that of unmodified F_1 due to the presence of NBD bound to lysine, which is not the case.

Finally, a higher than predicted reactivation can be obtained if modified and unmodified β-subunits have a preference for assembling into F_1 containing modified and unmodified β-subunits, respectively. Our present results do not fully rule out such a preference, although it is very unlikely that such a preference should produce the theoretical curve for a 2-site model, even after a second treatment of the F_1 with NBD-Cl and LiCl (see also Discussion in Ref. 15).

Since 2-azido-ATP can be bound tightly to a catalytic site [9,23], we also repeated the dissociation-association procedure with F_1 partially modified and inactivated with 2-nitreno-AT(D)P, covalently bound to a catalytic site. The illumination of 2-azido-ATP-F_1 was repeated after 30 min and 60 min because the tetrazolium form of the compound (approx. 55% under the experimental conditions, Ref. 24), which has the same affinity for F_1 as the azido-form [25], is not photoreactive and isomerises to the azido form only slowly [23,24]. As can be seen in Fig. 4, the dissociation-reconstitution procedure did not result in reactivation, in contrast to that with NBD-modified F_1. The reason for this lack of reactivation was found when samples taken after 100 s of incubation in the dissociation medium were put on polyacrylamide gels in the absence of SDS. With unmodified F_1 the band of native F_1 had largely disappeared, while the band at the position of the isolated

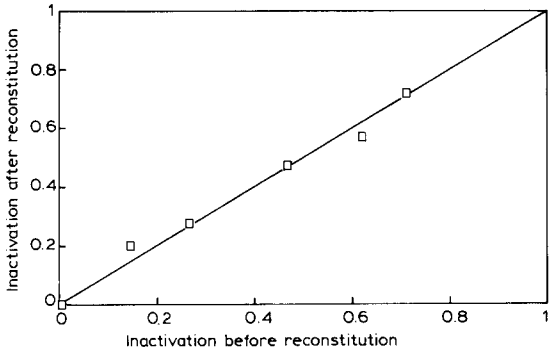

Fig. 4. Reactivation of F_1 modified by 2-nitreno-ATP. F_1 (4.1 mg/ml) was incubated with 1 mM 2-azido-ATP in medium B and after 10 min free and loosely bound 2-azido-ATP was removed by repeated centrifuge column gel filtration. The obtained F_1 preparation, containing 1 mol 2-azido-AT(D)P per mol F_1 (or the tetrazolium form of the compound) was illuminated to various degrees resulting in the formation of F_1 modified by 2-nitreno-AT(D)P. The partially inactivated samples were treated with LiCl and desalted, and the activities were measured before and after the dissociation-reconstitution procedure.

β-subunits was very intense, but with modified F_1 the residual F_1-band was about proportional to the level of modification (see Fig. 5). It is concluded from this experiment that covalent binding of 2-nitreno-ATP to a catalytic site protects the enzyme against dissociation and that, as suggested previously, bound adenine nucleotides contribute significantly to the stability of the mitochondrial F_1.

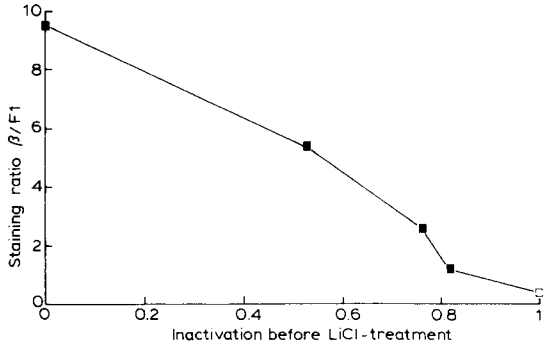

Fig. 5. Dissociation of 2-nitreno-AT(D)P-F_1. F_1, inactivated by covalent binding of 2-nitreno-AT(D)P to a catalytic site, was treated with 3 M LiCl and after 100 s samples were put on 4% polyacryalmide gels, without urea or SDS. After electrophoresis according to Ref. 18, staining and destaining, the staining of the bands of native F_1 and the β-subunit were compared (■). The point (□) represents the result obtained with intact F_1, not treated with LiCl.

Acknowledgements

The authors thank Prof. K. van Dam for reading the manuscript and Dr. M. van Dongen for valuable discussions. The work was partially supported by the Netherlands Organization In the Advancement of Pure Research (Z.W.O.) under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.).

References

1 Stutterheim, E., Henneke, M.A.C. and Berden, J.A. (1981) Biochim. Biophys. Acta 634, 271–278
2 Todd, R.D., Griesenbeck, R.A. and Douglas, M.G. (1980) J. Biol. Chem. 255, 5461–5467
3 Senior, A.E. and Wise, J.G. (1983) J. Membr. Biol. 73, 105–124
4 Harris, D.A., Rosing, J., Van de Stadt, R.J. and Slater, E.C. (1973) Biochim. Biophys. Acta 314, 149–153
5 Cross, R.L. and Nalin, C.M. (1982) J. Biol. Chem. 257, 2874–2881
6 Gresser, M.J., Myers, J.A. and Boyer, P.D. (1982) J. Biol. Chem. 257, 12030–12038
7 Esch, F.S. and Allison, W.S. (1979) J. Biol. Chem. 254, 10740–10746
8 Van Dongen, M.B.M. and Berden, J.A. (1986) Biochim. Biophys. Acta 850, 121–130
9 Van Dongen, M.B.M., De Geus, J.P., Korver, T., Hartog, A.F. and Berden, J.A. (1986) Biochim. Biophys. Acta 850, 359–368
10 Lötschen, H.R. and Capaldi, R.A. (1984) Biochem. Biophys. Res. Commun. 121, 331–339
11 Boekema, E.J., Berden, J.A. and Van Heel, M.G. (1986) Biochim. Biophys. Acta 851, 353–360
12 Kironde, F.A.S. and Cross, R.L. (1986) J. Biol. Chem. 261, 12544–12549
13 Slater, E.C., Berden, J.A. and Van Dongen, M.B.M. (1986) J. Protein Chem. 5, 177–192
14 Wang, J.H. (1984) Biochemistry 23, 6350–6354
15 Wang, J.H. (1985) J. Biol. Chem. 260, 1374–1377
16 Wang, J.H., Joshi, V. and Wu, J.C. (1986) Biochemistry 25, 7996–8001
17 Cross, R.L., Grubmeyer, C. and Penefsky, H.S. (1982) J. Biol. Chem. 257, 12101–12105
18 Knowles, A.F. and Penefsky, H.S. (1972) J. Biol. Chem. 247, 6617–6623
19 Steinmeier, R.C. and Wang, J.H. (1979) Biochemistry 18, 11–18
20 Berden, J.A. and Verschoor, G.J. (1978) Biochim. Biophys. Acta 504, 278–287
21 Verschoor, G.J. (1982) Structure of mitochondrial F_1 ATPase, Ph.D. Thesis, University of Amsterdam

22 Ferguson, S.J., Lloyd, W.J., Lyons, M. and Radda, G.K. (1975) Eur. J. Biochem. 54, 117–126
23 Van Dongen, M.B.M. and Berden, J.A. (1987) Biochim. Biophys. Acta 893, 22–32
24 Czarnecki, J.J. (1984) Biochim. Biophys. Acta 800, 41–51
25 Boulay, F., Dalbon, P. and Vignais, P.V. (1985) Biochemistry 24, 7372–7379