Vol. 99, No. 3, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1981 Pages 813-819

1-FLUORO-2,4-DINITROBENZENE MODIFIES A TYROSINE RESIDUE WHEN IT INACTIVATES THE BOVINE MITOCHONDRIAL F_1-ATPASE

William W. Andrews and William S. Allison

Department of Chemistry, M-001
University of California, San Diego
La Jolla, CA 92093

Received February 18,1981

SUMMARY: The prompt addition of dithiothreitol to a final concentration of 90 mM to the bovine mitochondrial F_1-ATPase inactivated with 1-fluoro-2,4-dinitrobenzene (FDNB) leads to recovery of 50-75% of the initial activity, depending on the conditions. Prior inactivation of the ATPase with FDNB decreases the ^{14}C bound by the enzyme when it is treated subsequently with 7-chloro-4-nitro[^{14}C]benzo-2-oxa-1,3-diazole (NBD-Cl) by about 0.7 g atom per mol. From these results it is concluded that the fraction of MF_1 inactivated by FDNB which is reversible by dithiothreitol is caused by the modification of the same tyrosine residue that is altered when NBD-Cl inactivates the enzyme.

INTRODUCTION: Some time ago it was shown that the modification of a single tyrosine residue per mol of MF_1[1] with NBD-Cl[1] leads to complete inactivation of the enzyme (1). Under slightly alkaline conditions the NBD-group was shown to migrate to a lysine residue in the β subunit and the enzyme remained inactive (2). Since MF_1 has at least two, and probably three copies of β, the catalytic subunit (3,4), the stoichiometry of inactivation with NBD-Cl is difficult to explain without invoking cooperativity between subunits. The function of the site that reacts with NBD-Cl became more mysterious when it was shown that a different tyrosine residue in the β subunit is modified when MF_1 is inactivated with the affinity reagent, FSBA[1] (3). Furthermore, the complete inactivation of MF_1 by [^{14}C]FSBA is accompanied by the modification

[1] Abbreviations: FDNB, 1-fluoro-2,4-dinitrobenzene; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; MF_1, the bovine mitochondrial F_1-ATPase; and FSBA, 5'-p-fluorosulfonylbenzoyladenosine.

0006-291X/81/070813-07$01.00/0

of the tyrosine residue for which it is specific in all copies of the β subunit (3,5).

It has been reported that MF_1 is also inactivated by FDNB[1], a reagent with structural and electrophilic properties very similar to those of NBD-Cl, and that this inactivation is not reversible by thiolysis (6). Recently it was reported that the rate of inactivation of MF_1 by [^{14}C]FDNB as well as the rate of ^{14}C incorporation that accompanied it were slowed by the prior blocking of the essential tyrosine residue with NBD-Cl (7). From these results it was concluded that the lysine residue modified by FDNB is the same one to which the NBD-group migrates under slightly alkaline conditions (7). This interpretation, if correct, means that a tyrosine residue in MF_1 reacts with NBD-Cl but not with FDNB, while a lysine residue that is adjacent to it reacts only with FDNB. Since such selective reactivity seems unlikely for these two reagents, an investigation was initiated to identify precisely the amino acid residues that are modified when MF_1 is inactivated by NBD-Cl and by FDNB.

MATERIALS AND METHODS: MF_1 was prepared by a procedure (5) which includes a combination of steps from methods described by Senior and Brooks (8) and by Knowles and Penefsky (9). The enzyme was assayed with the ATP regenerating system described by Vogel and Steinhart (10). Protein concentrations were determined by the Lowry method (11) with a corrected color value (12). Adenine nucleotides and other biochemicals were obtained from Sigma Chemical Co. FDNB, NBD-Cl, and 1,5-difluoro-2,4-dinitrobenzene were obtained from Pierce Chemical Co. [^{14}C]NBD-Cl was purchased from Research Products International. Radioactivity was determined in Amersham ACS using a Beckman LS 100 counter.

RESULTS AND DISCUSSION: Following the inactivation of MF_1 with FDNB at pH 8.0, the immediate addition of dithiothreitol to a final concentration of 90 mM leads to the slow recovery of enzyme activity illustrated in Fig. 1. About 50% of the initial activity is regained under the conditions described in Fig. 1. When similar experiments were conducted in the presence of ADP or 25% glycerol at pH 8.0, about 70% of the initial activity was recovered in the reactivation step as shown in Table I. Under none of these conditions did reactivation occur when dithiothreitol was added to the FDNB-treated enzyme at a final concentration of 10 mM for 5 minutes.

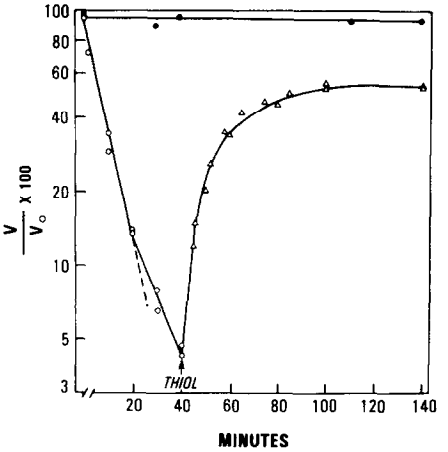

Figure 1. The inactivation of MF_1 by 1 mM FDNB and its subsequent reactivation by 90 mM dithiothreitol. To 0.3 mg of MF_1 in 0.50 ml of 50 mM triethanolamine-SO_4, pH 8.0 containing 4 mM EDTA, was added 5 µl of 100 mM FDNB in ethanol. After incubation for 40 min. at 23°C, 200 µl of the reaction mixtures were added to 20 µl of 1 mM dithiothreitol. Samples, 3-10 µl, of the inactivation and reactivation mixtures and untreated controls were assayed at the times indicated.

That part of the inactivation caused by FDNB which is reversed by 90 mM dithiothreitol cannot be due to the modification of a primary amino group (13) Although each of the derivatives formed when FDNB reacts with the side chains

TABLE I. THE INACTIVATION OF MF_1 BY FDNB AND ITS SUBSEQUENT REACTIVATION BY DITHIOTHREITOL UNDER VARIOUS CONDITIONS

Variation[a]	k_{inact} min^{-1}	Maximum Reactivation by Dithiothreitol (%)
None	0.099	52
4 mM ADP	0.077	75
4 mM ADP 4 mM $MgSO_4$	0.074	70
4 mM ATP	0.057	54
25% Glycerol 50 mM HEPES	0.112	69
50 µM 1,5-difluoro-2,4-dinitrobenzene[b]	0.202	25

[a] The reaction conditions are presented in the legend of Fig. 1 with the changes indicated here.

[b] Replaces 1 mM FDNB present in the other reaction mixture.

TABLE II. DECREASE IN [^{14}C]NBD-Cl BOUND AFTER INACTIVATION OF MF_1 WITH FDNB

MF_1, 0.24 mg in 200 µl of 50 mM triethanolamine-SO_4, pH 8.0, containing 4 mM EDTA was inactivated in the dark with 1 mM FDNB. Greater than 90% inactivation occurred in 1 hr. at which time the inactivated enzyme and an untreated control were subjected to centrifugation-elution (13) on columns of Sephadex G-50 that were equilibrated with 50 mM triethanolamine-SO_4 containing 4 mM EDTA at either pH 7.0 or 7.5. Both effluents in each experiment were treated with [^{14}C]NBD-Cl at a final concentration of 0.1 mM. When the controls lost greater than 90% activity, 100 µl of the reaction mixtures were subjected to centrifugation-elution on columns of Sephadex G-50 equilibrated with 50 mM Na-borate, pH 8.0. To the remaining of 100 µl of the reaction mixtures 1 µl of 1 M dithiothreitol was added. After 5 minutes 2 µl samples were assayed while the remainder of these sulutions was subjected to centrifugation-elution on columns equilibrated with Na-borate, pH 8.0. Samples of each effluent from which excess [^{14}C]NBD-Cl had been removed were counted while other samples were used for protein determinations.

Treatments Additional to [^{14}C]NBD-Cl	g atoms ^{14}C / mol MF_1		% Reactivation by 10 mM Dithiothreitol	
	pH 7.0	pH 7.5	pH 7.0	pH 7.5
A. None	1.40	1.43		
B. +DTT	0.52	0.78	91	72
C. +FDNB	0.71	0.63		
D. +FDNB; +DTT	0.43	0.50	0	0
A-C[a]	0.69	0.80		
(A-B)-(C-D)[a]	0.60	0.52		

[a]See the text for an explanation of these quantities.

of tyrosine, histidine, and cysteine residues are cleaved by thiols to regenerate the parent amino acids (13,14), evidence which follows suggests that the reversible part of the inactivation following treatment of MF_1 with FDNB is due to the modification of the same tyrosine residue that reacts with NBD-Cl. It is known that the modification of a single tyrosine residue per mol of MF_1 causes complete inactivation of the enzyme and that thiolysis of the nitroaryl ether derivative of the inactive enzyme regenerates nearly all of the activity (1). Table II summarizes binding experiments which show that prior inactivation of MF_1 with FDNB reduces the radioactivity bound to the enzyme when it is subsequently treated with [^{14}]NBD-Cl. The total amount of ^{14}C incorporation blocked by the prior inactivation of the enzyme is repre-

sented in Table II as the difference, A-C, and is about 0.7 g atom per mol of MF_1 in the two experiments. The difference, (A-B) - (C-D), shown in Table II represents the decrease in the amount of ^{14}C incorporated that can be removed by thiols caused by prior inactivation of the enzyme with FDNB. This value is about 0.55 g atoms per mol of MF_1 in the two experiments and probably represents that part of the ^{14}C incorporation that is due to the modification of tyrosine residues by [^{14}C]NBD-Cl. It is interesting that this value corresponds to the maximum amount of ATPase activity recovered when the FDNB inactivated enzyme is treated with 90 mM dithiothreitol as shown in Fig. 1.

That part of these inactivations by FDNB not reversed by dithiothreitol is probably due to the modification of primary amino groups. Functionally important lysines in F_1-ATPases have been implicated by chemical modification studies with pyridoxal phosphate (15-17). It is not known if the lysine residue to which the NBD-group migrates also reacts with pyridoxal phosphate. When the NBD-group migrates in MF_1, the nitroaryl ether derivative of the tyrosine residue that reacts initially with NBD-Cl is attacked by a lysine residue in the β subunit (2). To explain the inactivation and reactivation curves of Fig. 1, it might be suggested that FDNB reacts at roughly equal rates with either one or the other of these two residues, but never both in the same subunit during the inactivation. However, this interpretation is inconsistent with other observations. For instance as shown in Table I, ADP has little effect on the rate of inactivation by FDNB while it has a marked effect on the specificity. The converse of these effects is promoted by ATP. These observations and others (18) suggest that adenine nucleotides affect indirectly the inactivation of MF_1 by NBD-Cl.

Ting and Wang have proposed a catalytic mechanism for MF_1 which includes functional roles for both the tyrosine residue that reacts with NBD-Cl and the lysine residue to which the NBD-group migrates. The functional role assigned to the lysine residue in this mechanism is based on the protective effect of

ATP and Pi on the inactivation of MF_1 by FDNB (7). When considered with the evidence presented here it is obvious that the functional role assigned to the lysine residue in the proposed mechanism rests on ambiguous evidence. Furthermore, experimental evidence ignored by Ting and Wang, suggests that the assignment of a direct catalytic role to the tyrosine residue that reacts with NBD-Cl is incorrect (3). It has been shown that reversible blocking of the tyrosine residue in MF_1 with NBD-Cl does not affect the irreversible inactivation of the enzyme by [^{14}C]FSBA (3). This inactivation proceeds with the modification of a different tyrosine residue in all copies of the β subunit. Moreover, comparison of the rate of inactivation of the enzyme with FSBA as a function of pH with the pH-rate profile of the hydrolytic reaction strongly suggests that the conjugate base of the tyrosine residue which reacts with FSBA functions directly in catalysis (5).

ACKNOWLEDGEMENTS

This work was supported by the United States Public Health Service Grant GM-16979 and the National Science Foundation Grant PCM-12467.

References:

1. Ferguson, S.J., Lloyd, W.J., Lyons, M.H., and Radda, G.K. (1975) Eur. J. Biochem. 54, 117-126.
2. Ferguson, S.J., Lloyd, M.H., and Radda, G.K. (1975) Eur. J. Biochem. 54, 127-133.
3. Esch, F.S. and Allison, W.S. (1979) J. Biol. Chem. 254, 10740-10746.
4. Wagenvoord, R.J., Kemp, A., and Slater, E.C. (1980) Biochim. Biophys. Acta 593, 204-211.
5. Esch, F.S., and Allison, W.S. (1978) J. Biol. Chem. 253, 6100-6106.
6. Ferguson, S.J., Lloyd, W.J., and Radda, G.K. (1974) FEBS Letts. 38, 234-236.
7. Ting, L.P., and Wang, J.H. (1980) Biochemistry 19, 5665-5670.
8. Senior, A.E., and Brooks, J.C. (1970) Arch. Biochem. Biophys. 140, 257-266.
9. Knowles, A.F., and Penefsky, H.S. (1972) J. Biol. Chem. 247, 6624-6630.
10. Vogel, G., and Steinhart, R. (1976) Biochemistry 15, 208-216.
11. Lowry, O.H., Roseborough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
12. Penefsky, H.S. (1979) Methods Enzymol. 55, 304-308.
13. Shaltiel, S. (1967) Biochem. Biophys. Res. Commun. 29, 178-183.
14. Fridkin, M., Hazum, E., Tauber-Finkelstein, M., and Shaltiel, S. (1977) Arch. Biochem. Biophys. 178, 517-526.

15. Sugiyama, Y. and Mukohata, Y. (1978) FEBS Letts. 98, 276-280.

16. Godinot, C., Penin, F., and Gautheron, D.C. (1979) Arch. Biochem. Biophys. 192, 225-234.

17. Peters, H., Risi, S., and Dose, K. (1980) Biochem. Biophys. Res. Commun. 97, 1215-1219.

18. Ferguson, S.J., Lloyd, W.J., Radda, G.K., and Slater, E.C. (1976) Biochim. Biophys. Acta 430, 189-193.