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LIGAND-INDUCED VARIATIONS IN SUBUNIT ASSOCIATIONS IN BOVINE HEART F_1 ATPASE

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SUMMARY. Bovine heart soluble F_1 ATPase shows ligand dependent changes in subunit accessibility to the protein labelling reagents acetic anhydride and diazonium benzenesulphonic acid. These correlate with changes in the ATPase activity of the enzyme induced by the same ligands. In particular, NAD^+ and NADH show concentration dependent effects, the effect of the reduced nucleotide being opposite to that of the oxidised form.

The F_1 component of the ATPase of mitochondria from various sources is activated by oxy-anions, notable dichromate, borate, maleate and sulphite (1,2). In contrast free bivalent cations frequently inhibit activity (3,4). Activation and inactivation of F_1 by ligands may operate at the active centre by, for example, oxy-anions acting as Lewis bases as proposed by Pedersen (5). Regulatory sites that influence catalytic sites by changing the conformation in and between subunits may also be involved (6,7). The following work investigated conformational changes under defined conditions of activation and inactivation by monitoring subunit labelling by the radio chemical probes $[^{14}C]$ acetic anhydride and $[^{35}S]$ diazonium benzenesulphonic acid.

METHODS. Preparation of F_1: Bovine heart mitochondria were prepared (8) and sonicated (8 mins; 300 watt input; 25 mm probe) under ice cold conditions at 30 mg protein ml^{-1} in 250 mM sucrose, 10 mM Tris chloride at pH 7.5. The suspension was spun (15 mins 40,000g) and the supernatant recentrifuged (90 mins 80,000g). The pellet was suspended in the buffer to 20 mg protein ml^{-1} and sonicated (4 mins; 100 watt input; 15 mm probe) giving sub-mitochondrial particles. F_1 was prepared from SMP by the method of Knowles and Penefsky (9) and stored at 5°C at 5-10 mg protein ml^{-1} in 250 mM

ABBREVIATIONS:

MOPS : Morpholinopropane sulphonic acid

Butyl BPD : 2-(4'-tert-Butylphenyl)-5-(4''-biphenylyl)-1,3,4-oxadiazole

DABS : Diazonium benzenesulphonic acid

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sucrose, 50 mM Tris chloride, 2 mM EDTA, 4 mM ATP made up to 2 M ammonium sulphate (pH 8.0) Before experiments the preparation was desalted against the appropriate buffer, using an immersible molecular separator.
F_1 activity assays: The pH method of Mitchell and Moyle (10) was used. The buffer systems were 3.3 mM glycylglycine, 150 mM KCl or 10 mM morpholinopropane sulphonic acid, 150 mM KCl, both at pH 7.05.
Radiolabelling: The [^{14}C] acetic anhydride labelling technique was similar to that of Kagawa and Racker (11). 1-2 mg F_1 was desalted and preincubated in the appropriate buffer (1.5 ml) with the ligand under investigation. After 60 seconds [^{14}C] acetic anhydride in acetone (100 ul) was added. Labelling was quenched after 2 minutes by addition of 1.5 volumes of saturated ammonium sulphate. The precipitated protein was pelleted by Eppendorf microfuge (6 mins) and resuspended in dissociating buffer - 1% (w/v) sodium dodecylsulphate, 1% (w/v) mercaptoethanol, 10 mM H_3PO_4 adjusted to pH 6.8 with Tris base, for 30 minutes at 50°C prior to electrophoresis.
Labelling with [^{35}S] DABS was carried out in Dr. R.A. Capaldi's laboratory, University of Oregon, U.S.A. Samples containing 1 mg of partially desalted F_1 in 100 ul of 10 mM MOPS, 150 mM KCl, pH 7.05 were extensively dialysed against the same buffer for 1 hour 15 min. After a further 30 minutes with the ligand present 15 ul (11 uC) of [^{35}S] DABS in 200 mM potassium phosphate buffer, pH 8.0, was added for 30 minutes at room temperature. The reaction was quenched with 25 ul of 50 mM Tris chloride, 5 mg ml^{-1} histidine and dialysed against four changes of 500 ml of 1% (w/v) sodium dodecylsulphate 50 mM Tris chloride, 5 mM histidine, pH 7.6. After dialysis, 0.7 volumes of 10% (w/v) sodium dodecylsulphate, 5% (w/v) mercaptoethanol, 8 M urea were added to each sample to dissociate the enzyme for electrophoresis.

Electrophoresis: The Weber and Osborn (12) system was used with 10% acrilamide and a monomer:crosslinker ratio of 37:1. The gel contained 0.1% (w/v) sodium dodecylsulphate and the running buffer was 130 mM sodium phosphate, 0.1% (w/v) sodium dodecylsulphate, pH 7.3. 20-200 ug F_1 was applied to each rod gel. Calibration proteins were prepared in the same way as the F_1 samples and run concurrently. The gels were extracted and fixed (13). After staining with Coomasie Blue R250 (12) the gels were scanned, frozen and sliced into 1 mm sections using a Mickel slicer. Each slice was solubilised in 500 ul 500 mM NaOH, 5% (w/v) sodium dodecyl sulphate overnight at 60°C. 5.2 ml of scintillation fluid (0.7% butyl BPD in 2:1 toluene:triton X 100) were added and the samples counted, after standing for at least an hour to reduce any chemiluminescence.

ATP, MOPS, NAD^+, NADH and Tris were obtained from Sigma Chemical Company, Butyl BPD from Koch-Light and radiolabelled acetic anhydride from the Radiochemical Centre, Amersham, U.K. [^{35}S] DABS was gifted by Dr.R.A. Capaldi.

RESULTS AND DISCUSSION

Characteristics of the F_1 preparation:

The preparation method yielded, on average, 4.5 mg F_1 protein per g mitochondrial protein. The F_1 was over 99% pure as judged from non-dissociating gel electrophoresis (9) and had a specific activity of between 150 and 190 u moles ATP hydrolysed min^{-1} mg F_1 protein^{-1} at pH 7.05 and 25°C. The molecular weights of the subunits, as indicated by the Weber and Osborn (12) gel system, were A:61.2k, B:56.5k, C:33.8k, IF_1 (The inhibitor protein) :10.6k, E:9.0k.

Effects of adenine nucleotides on [^{14}C] acetic anhydride labelling.

Titration of F_1 and sub mitochondrial particles with the inhibitor protein IF_1 and related experiments (14,15,16) have led to proposals that, upon MgATP binding followed by ATP hydrolysis, F_1 assumes a new conformational state that facilitates the binding of IF_1. The resulting F_1-IF_1 complex inhibits enzyme activity, a state relieved in competent mitochondria by membrane energisation which supposedly dissociates the complex. Since IF_1 need not actually leave the enzyme for inhibition to be relieved (17) there may be separate inhibitory and non-inhibitory sites for IF_1 or it may bind in different modes to the same site. The classical method used in this paper for isolating F_1 leaves a substantial proportion of IF_1 still associated with the enzyme. It was therefore of interest to see if the proposed in situ conformational changes referred to above might be reflected in MgATP induced changes in the association of this IF_1 with the subunits of the F_1.

Table 1 shows that MgATP did, in fact, significantly change the labelling patterns of the subunits relative to those of controls without nucleotide and with MgADP. Under the conditions used in these and later experiments less than 0.2 mol acetic anhydride reacted with each mol of F_1 and the pH change in the medium was less than 0.05 units. At this concentration the label had no discernable effect on F_1 activity and no additional bands appeared in non-dissociating gel electrophoresis. MgATP led to IF_1 and D becoming significantly more accessible to acetylation and the larger subunits less so. However at present it is not prudent to interpret these results beyond the point that MgATP hydrolysis by isolated F_1 is linked to changes in the exposure of nucleophilic sites that indicate changes in F_1-IF_1 association.

Activating and inactivating ligands and [^{14}C] acetic anhydride labelling.

The inhibition of F_1 by free Mg^{2+}, Mn^{2+} and Ca^{2+} was very similar, 20 mM causing about 70% inhibition (Table 2). Supplementary experiments showed that inactivation by Mg^{2+} was particularly rapid, 90% of the max-

Table 1: Effects of incubating F_1 with adenine nucleotides on [^{14}C] acetic anhydride labelling of subunits. Buffer - 3.3 mM glycylglycine, 150 mM KCl, pH 7.05. Proportion of counts in each subunit expressed as percentage ± one standard deviation. Sample number in brackets.

	A/B	C	D	IF_1	E	Cpm 100 ug F_1^{-1}	Mol label mol F_1^{-1}
Control (17)	53.0 ±5.5	22.6 ±3.5	3.5 ±0.6	2.4 ±0.7	18.3 ±6.2	2,435	0.10
10 mM MgATP (7)	35.1 ±3.1	25.5 ±2.1	8.2 ±0.5	10.6 ±1.4	21.1 ±2.6	3,263	0.14
10mM Mg ADP (6)	51.1 ±1.6	20.5 ±0.6	3.0 ±0.3	4.3 ±0.3	21.2 ±1.4	3,192	0.13

imum inhibition occurring within 5 seconds. Various anions, sulphite, borate, maleate, bicarbonate and dichromate, were tested over a range of concentrations. Dichromate and borate were the only anions that had a significant activating effect. Figure 1 shows that at concentrations less than about 150 mM NAD^+ inhibits whereas at higher concentrations it activates. NADH produced the opposite effect, activating at low concentrations and inhibiting at higher ones. The nicotinamide nucleotides produced the most marked concentration dependent effects of any ligands investigated.

Table 2: Anion and cation effects on F_1 activity in MOPS and Glycylglycine buffers as defined in Methods. Enzyme preincubated for 60 sec with ligand before addition of ATP to 2.8 mM. Activity in u moles ATP hydrolysed min^{-1} mg F_1 $protein^{-1}$.

		Inactivating cations			Activating anions	
	Control	20mM $MgCl_2$	20mM $CaCl_2$	20mM $MnCl_2$	1mM $K_2Cr_2O_7$	5mM Na_2BO_3
MOPS buffer	137.0 ±4.4(4)	42.1 ±1.2(3)	42.9 ±5.0(3)	40.6 ±3.0(3)	-	-
	149.3 ±6.1(6)	-	-	-	177.4 ±2.4(3)	-
	162.2 ±4.6(3)	*52.9 ±6.1(3)	-	-	210.9 ±10.3(3)	200.3 ±8.2(3)
Glycyl-glycine buffer	183.8 ±5.3(3)	-	-	-	237.7 ±15.6(3)	204.6 ±12.1(3)
	176.2	53.6	62.7	-	211.2	-
Mean % change		-68.8	-66.1	-70.4	+24.5	+17.3

* indicates 10 mM $MgCl_2$.

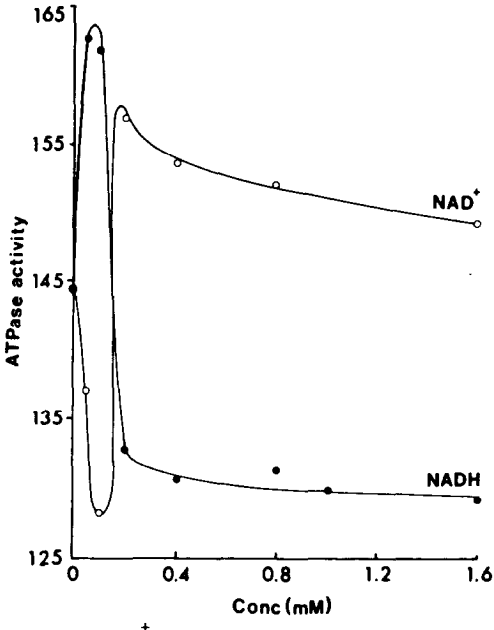

Figure 1: Effects of NAD^+ and NADH on ATPase activity in MOPS buffer. Conditions as in legend to Table 2.

Table 3 shows a trend towards a common pattern of changes in reactivity of subunits towards acetylation in the presence of the activating ligands, dichromate, 100 uM NADH and 1 mM NAD^+. These all decreased the proportion of label in the D and E subunits, increased it in IF_1 and left the proportion in A/B and C relatively unchanged. In contrast the inactivating ligands, Mg^{2+} and 1 mM NAD^+ increased the proportion of label in D and E and tended to decrease it in A/B and C. Most notably they increased greatly the proportion in IF_1.

These labelling differences reflect variations in the accessibility of nucleophilic groups to acetylation, predominantly lysine although tyrosine, cysteine and serine are also attacked. Since access is mainly influenced by shielding effects the results suggest subunit association/dissociation phenomena related to ligand-induced activation and inactivation. In future it may be possible to define primary and secondary effects of the ligands by rapid kinetic studies and the orientation of the subunits in these particular conformations by peptide fingerprinting. IF_1 becomes more accessible to acetylation under both activating and inactivating conditions, although considerably more so in the latter case. This does not necessarily mean

Table 3: Effects of incubating F_1 with activating and inactivating ligands on [^{14}C] acetic anhydride labelling of subunits. Buffer - 10 mM MOPS, 150 mM KCl, pH 7.05. Counts expressed as percentages ± one standard deviation.

	A/B	C	D	IF_1	E	Cpm 100 ug F_1^{-1}	Mol label mol F_1^{-1}
Control (14)	74.4 ±2.7	15.9 ±1.0	3.8 ±0.7	1.5 ±0.5	4.4 ±0.8	144,760	0.16
1 mM $K_2Cr_2O_7$ (7) (activating)	77.3 ±2.7	13.7 ±1.1	3.4 ±0.2	2.2 ±0.4	3.4 ±0.9	143,690	0.16
100 uM NADH (7) (activating)	79.1 ±1.5	13.7 ±0.6	2.6 ±0.2	4.0 ±0.3	0.7 ±0.2	53,130	0.06
1 mM NAD^+ (8) (activating)	76.2 ±1.0	18.4 ±0.4	1.6 ±0.2	2.8 ±0.2	1.0 ±0.3	196,380	0.22
10 mM $MgCl_2$ (8) (inactivating)	57.5 ±2.2	14.8 ±1.6	4.8 ±0.2	11.5 ±0.8	12.0 ±1.2	153,118	0.17
1 mM NADH (8) (inactivating)	68.3 ±2.6	9.8 ±0.6	5.1 ±0.7	11.4 ±1.2	5.5 ±0.5	111,900	0.12

than the polarity of movement of IF_1 is the same in both situations. The orientations could be quite different. The proportionally greater acetylation of IF_1 by the inactivating ligands in Table 3 is consistent with the results for ATP and the evidence that it causes IF_1 to bind in an inhibitory mode (17).

Activating and inactivating ligands and [^{35}S] DABS labelling.

Activating and inactivating concentrations of NADH produced different distributions of [^{35}S] DABS labelling among the subunits (Table 4). At activating concentrations (200 uM) changes relative to the control were small with possible increases in the proportion of label in C and D and decreases in IF_1 and E subunits. Inactivating levels of NADH (1 mM) gave a different pattern. The A/B subunit proportion decreased significantly while that of C increased 2.5 fold. IF_1 became relatively four times more reactive and E became four times less reactive.

Although acetic anhydride and DABS both label nucleophilic side chains they differ in other characteristics. DABS is much more polar than acetic anhydride and is therefore less likely to attack groups within the hydrophobic

Table 4: Effects of incubating F_1 with activating and inactivating concentrations of NADH on [^{35}S] DABS labelling of subunits. Buffer - 10 mM MOPS, 150 mM KCl, pH 7.05. Results as percentages of label per subunit.

	A/B	C	D	IF_1	E	Cpm 100 ug F_1^{-1}
Control (2)	73.6 +0.5	14.5 +1.1	1.3 +0.6	3.2 +0.9	7.6 +0.1	30,302
200 uM NADH (activating)	73.6	18.2	2.1	1.8	4.3	5,459
1 mM NADH (inactivating)	45.3	36.7	1.8	14.7	1.6	3,382

interior of the enzyme. Therefore these results with [^{35}S] DABS supplement the data on [^{14}C] acetic anhydride labelling and confirm marked changes in the associations of the subunits related to enzyme activation and inactivation. The concentration dependent effects of the nicotinamide nucleotides are intriguing and could provide an extremely sensitive form of regulation of enzyme activity if operative in vivo. Control by the NADH/NAD^+ ratio, possibly mediated through the F_1-IF_1 system, could effectively link enzyme activity with the energy status of the mitochondrion.

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