PHOTOLABELLING OF MITOCHONDRIAL NADH DEHYDROGENASE WITH ARYLAZIDOPHOSPHATIDYLCHOLINE

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1. Introduction

The proton-translocating NADH dehydrogenase of bovine heart mitochondria has been isolated in substantially unmodified form as complex I or the NADH–ubiquinone oxidoreductase complex [1]. The isolated enzyme is a lipoprotein consisting of at least 26 different polypeptides [2] which bind a molecule of FMN and at least 5 iron–sulphur centres [3], 4 of which have been purified or partially purified [4]. Studies on the organisation of the constituent polypeptides both in the isolated enzyme and in the membrane have been carried out using hydrophilic probes such as diazobenzenesulphonate (DABS) [5] and the hydrophobic probe, iodonaphthylazide (INA) [6]. Objections to the use of INA have been raised [7] because of the rather polar nature and long lifetime of the photogenerated nitrene. Thus, it is possible that INA preferentially labels proteins at the membrane surface rather than those embedded in the hydrophobic region of the membrane although this may, in fact, not occur [8]. A potentially better membrane probe is a chemically reactive phospholipid analogue. Here, we have used a phosphatidylcholine with 12-amino-*N*-(2-nitro-4-azidophenyl) dodecanoic acid in the 2 position [9] to photolabel isolated complex I.

2. Materials and methods

Complex I was prepared as in [10]. Lipid-depletion and lipid-supplementation of the enzyme were as in [11] and [6], respectively. [^{3}H]Arylazidophosphatidylcholine (AAPC) was synthesised as in [12] with a specific radioactivity of 7.5 Ci/mmol. An ethanolic solution of AAPC was added to complex I (10 mg/ml 0.67 M sucrose/50 mM Tris–HCl (pH 8.0)) to a final concentration of 1% of the total phospholipid (~2 mol AAPC/mol enzyme). The final ethanol concentration was <2% (v/v). After 10 min at 0°C, the sample was diluted 5-fold with sucrose–Tris buffer and either photolysed directly or photolysed after overnight dialysis against 100 vol. of the same buffer. Complex I as prepared is soluble because of residual cholate. Incorporation of AAPC was therefore due to cholate-mediated fusion between complex I/lipid/cholate micelles and AAPC/cholate micelles followed by removal of detergent either by dialysis or dilution. Photolysis and determination of protein-bound radioactivity were carried out as in [6] except that samples were illuminated in plastic cells. Gel electrophoresis was conducted in 13–16.5% (w/v) acrylamide gradient gels using the Laemmli [13] buffer system or in 12.5% (w/v) acrylamide cylindrical gels using the Weber and Osborn system [14]. Radioactivity in gels was determined as in [6] or by fluorography using En^{3}Hance as the scintillant. Molecular masses of constituent polypeptides were determined by reference to M_r standards from BDH Chemicals, Poole.

3. Results and discussion

Under our conditions, incorporation of radioactivity into protein reached 50% of maximum after 30 s illumination. The time course and extent of labelling was the same whether the sample had been dialysed or diluted before illumination. A standard illumination period of 2 min was chosen. Over this period there was no loss of NADH-ubiquinone oxidoreductase activity. Fig.1 shows the distribution of radioactivity between complex I polypeptides after photolabelling in the absence (b) or presence (c) of SDS. The labelling of the intact enzyme is quite selective, being mainly confined to low M_r polypeptides. In

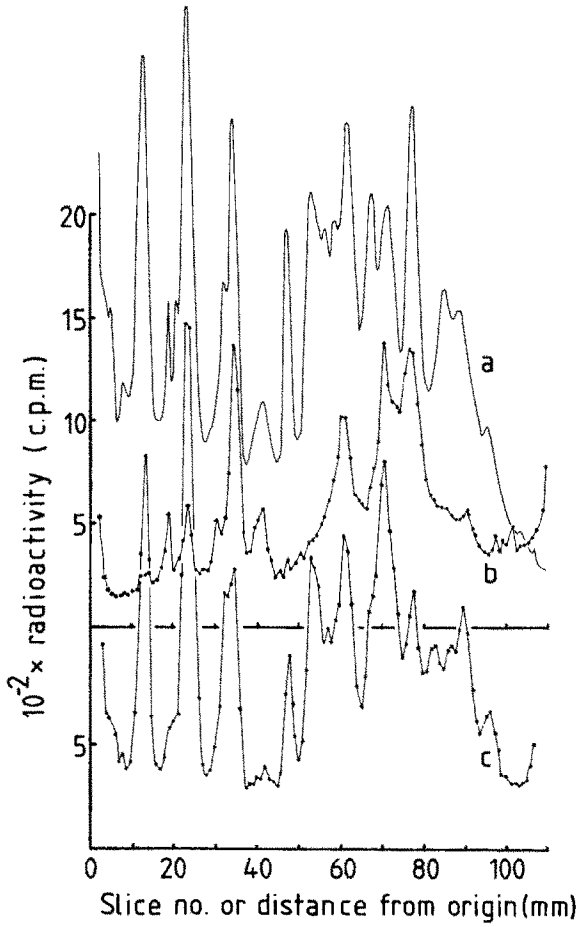

Fig.1. Labelling of complex I and SDS-treated complex I by AAPC. Complex I was labelled with AAPC in the absence and presence of 2% (w/v) SDS. Samples were precipitated by addition of cold, absolute ethanol and analysed by electrophoresis according to Weber and Osborn [14]. (a) Densitometer profile of complex I gel; (b) radioactivity incorporated into complex I; (c) radioactivity incorporated into SDS-treated complex I.

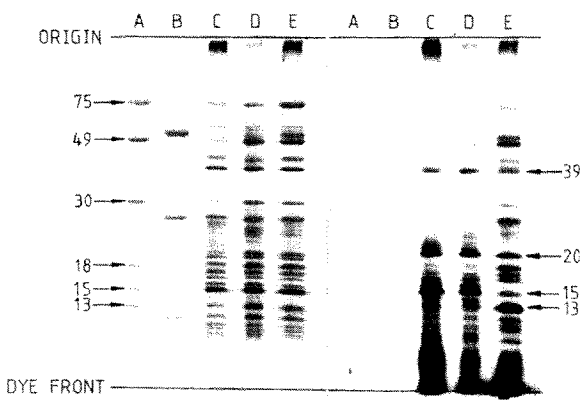

Fig.2. Labelling of complex I and subfractions by AAPC. Complex I labelled with AAPC was resolved with $NaClO_4$ and fractionated with ammonium sulphate [15]. Samples were analysed by electrophoresis on gradient gels and fluorography. (A) 'Iron–protein' fraction from 45 μg of complex I; (B) 'flavoprotein' fraction from 90 μg complex I; (C) insoluble residue from 45 μg complex I; (D) complex I (45 μg protein); (E) complex I (45 μg protein) treated with SDS before labelling. (Left) stained gel; (right) fluorograph. M_r values ($\times 10^{-3}$) of the 'iron–protein' subunits and of the major labelled polypeptides are given for reference.

contrast, SDS-treated enzyme was labelled in all polypeptides to approximately the same extent. Presumably, AAPC can associate equally well with all SDS–protein complexes. The incorporation of label into protein shown in fig.1(b) was ~8% of that added to the enzyme. Better resolution was obtained using gradient gels and fluorography as shown in fig.2. After photolabelling, complex I (D) was resolved with the chaotropic agent, $NaClO_4$, and the iron-protein (A) and flavoprotein (B) fragments were isolated [2,15]. The latter consists of 3 polypeptides [16] and catalyses NADH oxidation by a variety of electron acceptors [15]. The 2 larger subunits are iron–sulphur proteins [4]. The 'iron–protein' fraction contains at least 2 iron–sulphur centres [4] and contains several polypeptides as well as small amounts of the 'flavoprotein' fraction. Our M_r values of the smaller complex I polypeptides in [2,17] appear to be too high (e.g., [16]) and we have redetermined them using the gradient gel system. Our M_r values for the 'flavoprotein' fraction subunits are now in good agreement with [16] and the revised M_r values of the 'iron–protein' subunits are 75 000, 49 000, 30 000, 18 000, 15 000 and 13 000 as shown in fig.2. Very little radioactivity was found in any polypeptides of the 'iron–protein' or 'flavoprotein' fragments (fig.2). This was confirmed using cylindrical gels and detection of radioactivity in gel slices by scintillation counting. The only polypeptides to show any labelling were the 49 000 and 15 000 M_r polypeptides of the 'iron–protein' fraction and these only to a very small extent. Therefore, in agreement with our conclusions from INA labelling, the 'iron–protein' and 'flavoprotein' fragments have little if any contact with the membrane phospholipid. The insoluble residue from $NaClO_4$-resolved complex I (C) probably contains only one of the 5 or 6 iron–sulphur centres of complex I, but comprises 70% of the original com-

plex I protein [4]. The heavy labelling of several polypeptides of this fraction indicates extensive contact with the membrane phospholipid. The most heavily labelled polypeptides, of M_r 39 000, 20 000, 15 000 to 16 000, 13 000 and below, are the same as those labelled by INA, taking into account the superior resolution of the gel system of fig.2. However there were differences in the relative degrees of labelling. For example, the 39 000 M_r polypeptide was labelled relatively more heavily by AAPC while the opposite was true of the 15 000 M_r polypeptides (M_r = 18 000 in [6]). Since the chemically reactive group is very similar in both AAPC and INA, these differences may be ascribed to different locations of the 2 probes in the membrane. The reactive group of AAPC is presumably located in the middle of the bilayer while INA may be less constrained and could label proteins in a range of depths in the membrane. Thus, the 39 000 M_r polypeptide may be in contact with the membrane phospholipid only near the centre of the membrane. In support of this idea, the 39 000 M_r polypeptide is inaccessible to hydrophilic labels when the enzyme is in the mitochondrial membrane [5].

The INA-labelling profile of lipid-depleted complex I showed several differences compared with the undepleted enzyme [6]. These differences were also seen using AAPC as the label (fig.3). Thus, polypeptides of M_r 49 000, 42 000, 17 000, 13 000, 10 000, 7000 and 5000 showed extensive increase in labelling

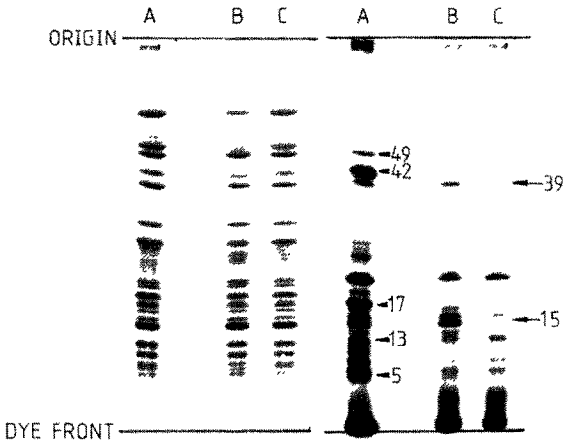

Fig.3. Effects of lipid-depletion or lipid-supplementation on AAPC labelling of complex I. Sample analysis and data presensation were as in fig.3. (A) Lipid-depleted complex I (60 μg protein); (B) native complex I (45 μg protein); (C) lipid-supplemented complex I (45 μg protein). M_r values ($\times 10^{-3}$) of some polypeptides showing changes in extent of labelling are given for reference.

(A). However, increasing the lipid content of the complex I caused decreases in the labelling of some polypeptides, for example, those of M_r 39 000 and 15 000 (C). As discussed in [6], these differences may be a result of conformational changes in the enzyme, particularly on depletion of lipids, or they be due to non-random distribution of different phospholipids, for example, cardiolipin and phosphatidylcholine. We cannot yet distinguish between these possibilities.

These results confirm those obtained with INA [6] and support the conclusion that the flavin and most of the iron–sulphur proteins of complex I are prevented from contact with the membrane phospholipid by a shell of small hydrophobic proteins.

Acknowledgements

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