Relative Rates of Turnover of Subunits of Mitochondrial Proteins

By JOHN H. WALKER, ROWLAND J. BURGESS and R. JOHN MAYER

*Department of Biochemistry, University of Nottingham Medical School,
Queen's Medical Centre, Nottingham NG7 2UH, U.K.*

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1. The double-isotope concept [Arias, Doyle & Schimke (1969) *J. Biol. Chem.* **244**, 3303-3315] for the measurement of protein turnover was used to estimate the turnover rates of protein subunits from rat liver submitochondrial fractions resolved by means of polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. $\text{NaH}^{14}\text{CO}_3$ and $[5\text{-}^3\text{H}]\text{arginine}$ were used as first and second precursors respectively. 2. Marked heterogeneity of protein subunit turnover rates is seen for protein subunits from water-soluble, salt-soluble and Tween 20-soluble mitochondrial proteins. 3. Much lower heterogeneity is seen in the turnover of protein subunits in Triton X-100-soluble material not binding to DEAE-cellulose at low ionic strength. The relative rates of turnover of proteins in this fraction are lower than for proteins in any other submitochondrial fraction. This fraction contains the integral membrane proteins. 4. Incorporation of $[^3\text{H}]\text{arginine}$ into subunits of the cytochrome oxidase complex is greatest for subunits with molecular weights in excess of 20000. 5. No correlation is seen between protein subunit size and the rate of turnover of the protein subunits in any of the submitochondrial fractions.

Relatively little is known about the mechanism of turnover of mitochondrial proteins. Fletcher & Sanadi (1961) measured the turnover of mitochondrial lipid and protein with $[^{14}\text{C}]\text{acetate}$ and $[^{35}\text{S}]\text{methionine}$ as the respective precursors. They found similar rates of turnover of lipid and protein, and suggested that mitochondria may turn over as a single unit.

Later studies (Brunner & Neupert, 1968; Druyan *et al.*, 1969; Gross *et al.*, 1969; DeBernard *et al.*, 1969; Aschenbrenner *et al.*, 1970; Ashwell & Work, 1970; Ip *et al.*, 1974; Satav *et al.*, 1976) have shown that mitochondrial proteins turn over at heterogeneous rates.

Interpretation of experimental data with regard to the mechanism of mitochondrial turnover depends on the reliability of the methods used to measure protein turnover rates, i.e. on the extent of reutilization of the chosen precursors (Swick & Ip, 1974). Thus the homogeneous mitochondrial turnover rates seen by Fletcher & Sanadi (1961) were probably a consequence of reutilization of radiolabelled precursors.

We have designed double-isotope experiments (Burgess *et al.*, 1978), based on the concept of Arias *et al.* (1969), to minimize first-precursor reutilization by using $\text{NaH}^{14}\text{CO}_3$ as the first precursor, and have used this technique to measure the rates of turnover of proteins in submitochondrial fractions from rat liver. The results indicated that proteins in different submitochondrial fractions had similar turnover rates. However, each fraction contains a mixture of proteins and therefore the isotope ratio of each fraction may reflect the average turnover rate of the proteins in that fraction.

In the experiments reported here we have used the same double-isotope procedure and have subsequently analysed the proteins in mitochondrial subfractions and in the cytochrome oxidase complex by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. Therefore the relative turnover rates of protein subunits have been estimated.

Additionally we have investigated the putative correlation between the subunit size of mitochondrial proteins and their turnover rate.

Materials and Methods

Materials

All analytical-reagent-grade chemicals were obtained from BDH Chemicals, Poole, Dorset, U.K., Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K., or Fisons, Loughborough, Leics., U.K. Sodium dodecyl sulphate (specially pure) was purchased from Fisons. Acrylamide (specially purified for electrophoresis) was obtained from BDH Chemicals. $\text{NaH}^{14}\text{CO}_3$ and $\text{L-[5-}^3\text{H]arginine monohydrochloride}$ were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Preparation of submitochondrial fractions

Submitochondrial fractions were prepared as described in the preceding paper (Burgess *et al.*, 1978).

Immunoabsorbent chromatography of cytochrome oxidase

An immunoabsorbent column (2.1 cm × 5.8 cm of packed gel, 20 ml bed volume) to cytochrome oxidase was prepared by the method of Walker & Mayer (1977). The immunoabsorbent was equilibrated with 20 mM-potassium phosphate, pH 7.0, containing 1% (w/v) Triton X-100 and 0.15 M-NaCl.

Phenylmethanesulphonyl fluoride (0.7%, w/v) in 95% (v/v) ethanol was added to each cytochrome oxidase preparation (final concentration 2 mM) before immunoabsorbent chromatography. Each preparation was applied to the immunoabsorbent column and left at room temperature for 2½ h. Unbound protein was eluted with 80 ml of 20 mM-potassium phosphate, pH 7.0, containing 1% (w/v) Triton X-100 and 0.15 M-NaCl. The column was then washed with 120 ml of 20 mM-potassium phosphate, pH 7.0, containing 0.15 M-NaCl before elution of the cytochrome oxidase with 80 ml of 8 M-urea containing 2% (w/v) sodium dodecyl sulphate. The eluate was dialysed extensively against warm running tap water in a rocking dialyser and then against distilled water (10 litres) before being freeze-dried. The freeze-dried solid was stored at -20°C.

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate

Two electrophoretic systems were used.

System of Weiss et al. (1971)

Slab gels (0.3 cm × 11 cm × 14 cm) were prepared in a Bio-Rad Dual Vertical Slab Gel Electrophoresis Cell (model 220) by the method of Weiss *et al.* (1971). The final acrylamide concentration was 10% (w/v) and the acrylamide/bisacrylamide ratio was 37:1.

System of Poyton & Schatz (1975)

The buffer systems of Poyton & Schatz (1975) were used to prepare a slab gel consisting of a lower 15% (w/v) polyacrylamide running gel (0.3 cm × 10 cm × 14 cm) and an upper, 2.5% (w/v) stacking gel (0.3 cm × 1 cm × 14 cm).

Preparation of samples for electrophoresis

Samples containing K⁺ were dialysed extensively against distilled water and then freeze-dried. Fractions containing the non-ionic detergents Tween 20 and Triton X-100 were precipitated by addition of 10% (w/v) trichloroacetic acid (final concentration 5%). Detergent was then removed by washing the precipitate with diethyl ether.

Freeze-dried or precipitated samples were taken up in 0.3–0.5 ml of sample buffer, and immediately boiled for 2 min. The Weiss sample buffer contains 10 mM-Tris/HCl, pH 8.0, and 2% (v/v) 2-mercaptoethanol. The Poyton & Schatz sample buffer contains 10 mM-sodium phosphate, pH 7.0, and 2% (v/v) 2-mercaptoethanol. Sodium dodecyl sulphate was included in each sample buffer at a concentration sufficient to give a ratio of sodium dodecyl sulphate/protein of at least 4:1 (w/w).

Electrophoresis procedure

For both systems 3 mg of each sample (0.3–0.5 ml) prepared as described above was mixed with 0.1 ml of glycerol containing 0.003% Bromophenol Blue. The mixture was then pipetted into a 3.4 cm-wide sample well in the gel surface. Electrophoresis was carried out at 40 mA per gel until the Bromophenol Blue marker reached the bottom of the gel (Weiss system) or until a cytochrome *c* protein marker had migrated 75–80% of the length of the gel (Poyton & Schatz system). Bovine serum albumin, ovalbumin and cytochrome *c* were used as protein standards.

Measurement of radioactivity in gels

Each gel was cut transversely into 2 mm strips, which were placed in scintillation vials and solubilized by incubating with 0.5 ml of H₂O₂ (100-volume) at 60°C for 16 h. Formic acid (0.5 ml; 90%, w/v) and 10 ml of a scintillant containing Triton X-100 (33% v/v), xylene (67%, v/v), 2,5-diphenyloxazole (0.55%, w/v) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.01%, w/v) were then added to each vial. Samples prepared in this way were left in the dark for at least 2 days. Radioactivity measurements and computations were carried out as described in the preceding paper (Burgess *et al.*, 1978).

Staining of gels

Protein standards were detected on gels by staining overnight in a solution of Coomassie Brilliant Blue (0.25%, w/v), methanol (45%, v/v) and acetic acid (10%, v/v). Gels were then destained by using repeated changes of a solution containing methanol (5%, v/v) and acetic acid (7.5%, v/v).

Results

Samples of submitochondrial fractions were subjected to polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. Fig. 1 shows the isotope ratios (³H/¹⁴C) for analyses of proteins in mitochondrial subfractions prepared as described in the preceding paper (Burgess *et al.*, 1978) for sequential slices of the gels. In two preliminary

experiments the isotope-ratio profiles of the sub-mitochondrial fractions were similar to those in Fig. 1.

Isotope ratios for protein subunits in the water-soluble fraction

Electrophoresis of the proteins in the fraction (which contains 52% of mitochondrial protein;

Burgess *et al.*, 1978) gave the isotope-ratio profile shown in Fig. 1(a). Isotope ratios for protein subunits in the preparation obtained from the liver of the test animal show a marked heterogeneity. In comparison, the theoretically expected similar ratios for the protein subunits in the preparation from the liver of the control animal were obtained. This was seen for all mitochondrial subfractions from control animals (Figs. 1b–1e).

Fig. 1. Analysis of the proteins in submitochondrial fractions by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate

Water-soluble (a), salt-soluble (b) and Tween 20-soluble material (c) and Triton X-100-soluble material binding to DEAE-cellulose (d) were analysed on 10% (w/v) polyacrylamide gels. Triton X-100-soluble material not binding to DEAE-cellulose (e) was analysed on 15% (w/v) polyacrylamide gels. Approx. 3 mg of protein was applied to each gel. The radioactivity in each gel slice was (a) 20–200 d.p.m. for ^{14}C and 300–2000 d.p.m. for ^3H , (b) 30–300 d.p.m. for ^{14}C and 300–3000 d.p.m. for ^3H , (c) 20–350 d.p.m. for ^{14}C and 200–4000 d.p.m. for ^3H , (d) 20–400 d.p.m. for ^{14}C and 300–3000 d.p.m. for ^3H , (e) 40–300 d.p.m. for ^{14}C and 200–3000 d.p.m. for ^3H . Protein standards are bovine serum albumin (BSA), ovalbumin (OV) and cytochrome c (CYT-C). Results are shown for protein subunits in the test fraction (●) and control fraction (Δ). Linear-regression analysis was used to obtain the straight lines for the data on protein subunits from control animals.

Isotope ratios for protein subunits in the salt-soluble fraction

Fig. 1(b) shows electrophoresis of the proteins in this fraction (which contains approx. 11% of mitochondrial protein; Burgess *et al.*, 1978). Isotope ratios for the protein subunits in preparations obtained from the liver of the test animal again show a marked heterogeneity.

Isotope ratios for protein subunits in the Tween 20-soluble fraction

Fig. 1(c) shows electrophoresis of proteins in the fraction (which contains approx. 11% of mitochondrial protein; Burgess *et al.*, 1978). Considerable heterogeneity in the turnover rates is again demonstrated for protein subunits in this fraction.

The results in Figs. 1(a)–1(c) show that some groups of protein subunits have much faster turnover rates than the average value for proteins in the whole fraction (see preceding paper: Burgess *et al.*, 1978).

Isotope ratios for protein subunits in material not binding to DEAE-cellulose

Fig. 1(d) shows electrophoresis of the proteins in this fraction (which contains approx. 9% of mitochondrial protein; Burgess *et al.*, 1978). Although the isotope ratios for protein subunits in the preparation from the liver of the test animal indicate some heterogeneity of protein subunit turnover, this is much less marked than for the other mitochondrial fractions.

Isotope ratios for proteins in material binding to DEAE-cellulose

The results in Figs. 1(a)–(d) were obtained with preparations from the livers of two animals. All the material that bound to DEAE-cellulose was used for the immunisolation of cytochrome oxidase. In a separate experiment (Experiment 2) with two more animals very similar isotope ratios were observed for protein subunits in submitochondrial fractions. However, in this experiment the isotope ratios for the protein subunits in material binding to DEAE-cellulose (half of which is cytochrome oxidase) were measured. The results (Fig. 1e) again show heterogeneity in isotope ratios for the protein subunits. In Experiment 2 half the amount of $\text{NaH}^{14}\text{CO}_3$ that was used in the first experiment was injected into the test and control animals.

In Figs. 1(a)–1(e) no clear indication of a relationship between isotope ratio and subunit size could be seen. Furthermore the results in Fig. 1(d) in comparison with those in Figs. 1(a), 1(b), 1(c) and 1(e) indicate low and relatively similar rates of turnover for protein subunits of different size.

Incorporation of [^3H]arginine into the subunits of immunisolated cytochrome oxidase

Very low ^{14}C radioactivity was present in the subunits of immunisolated cytochrome oxidase. Therefore it was not possible to measure isotope ratios for subunits of the enzyme. The incorporation of [^3H]arginine (in 4h) into protein subunits of immunisolated cytochrome oxidase is shown in Fig. 2. The largest incorporation of radioactivity is seen in the larger protein subunits (i.e. mol.wt. greater than approx. 20000).

Discussion

The results presented in the preceding paper (Burgess *et al.*, 1978) suggest that proteins in different submitochondrial fractions turn over at similar rates. However, Figs. 1(a)–1(e) reveal a marked heterogeneity in the isotope ratios of protein subunits in these submitochondrial fractions. The results indicate that within a given submitochondrial fraction there is heterogeneity in the rates of protein turnover. The similar rates of turnover previously observed for proteins in different subfractions are a consequence of measuring an average rate of turnover for a mixed population of proteins. The outcome of both these studies would not support the concept of homogeneous mitochondrial turnover (Fletcher & Sanadi, 1961).

Gear (1970) has proposed that the mitochondrial matrix and a 'primary' outer membrane turn over together with the inner membrane. Significant differences are observed for the isotope-ratio profiles for protein subunits (Fig. 1a) in the water-soluble

Fig. 2. Analysis of the incorporation of [^3H]arginine into protein subunits of immunisolated cytochrome oxidase by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate

Approx. 1–2mg of protein was analysed on 15% (w/v) polyacrylamide gels. Radioactivity in each gel slice was 35–400 d.p.m. for ^3H . Positions of subunits of protein standards are indicated. Protein standards are bovine serum albumin (BSA), ovalbumin (OV) and cytochrome c (CYT-C).

fraction (which contains proteins from the mitochondrial matrix and inter-membrane space) and for the Triton X-100-soluble material not binding (Fig. 1d) and binding (Fig. 1e) to DEAE-cellulose (which contains integral inner- and outer-membrane proteins). This indicates considerable differences in the turnover of proteins in the matrix and the mitochondrial membrane systems. It has previously been shown that some matrix proteins turn over independently of the mitochondrial membrane systems, as exemplified by the rapid rates of turnover of δ -aminolaevulinate synthetase (Tschudy *et al.*, 1965; Aschenbrenner *et al.*, 1970), ornithine aminotransferase (Ip *et al.*, 1974) and glutamate dehydrogenase (Nicoletti *et al.*, 1977).

The isotope ratios for protein subunits in the salt-soluble (Fig. 1b) and Tween 20-soluble (Fig. 1c) protein fractions (which might be expected to include some peripheral membrane proteins) indicate considerable heterogeneity in protein turnover rates. Furthermore the isotope-ratio profiles are very different, indicating large differences in the turnover of the proteins solubilized by these procedures.

A number of turnover studies have suggested a correlation between rate of turnover and protein subunit size for rat liver soluble and membrane proteins (Dehlinger & Schimke, 1971, 1972; Dice & Schimke, 1972; Dice *et al.*, 1973; Wang & Touster, 1975; Dean, 1975). In this study no such correlation can be seen in any of the submitochondrial protein fractions (Figs. 1a–1e).

The least heterogeneity in isotope ratios is shown for protein subunits in Triton X-100-soluble material not binding to DEAE-cellulose. The isotope ratios for protein subunits in the preparation obtained from the test animal are also minimally different from the isotope ratios in the preparation obtained from the control animal. This submitochondrial fraction contains most of the integral membrane proteins. The results show that integral mitochondrial membrane proteins turn over at more similar rates and at lower rates than any other proteins resolved by the solubilization procedures.

It has previously been shown that a number of inner-membrane proteins have similar rates of turnover (Druyan *et al.*, 1969; Gross *et al.*, 1969; Aschenbrenner *et al.*, 1970). Outer-membrane proteins are degraded at a higher rate than proteins of the inner membrane (Brunner & Neupert, 1968; DeBernard *et al.*, 1969; Druyan *et al.*, 1969). Further evidence that the inner membrane or a significant part of it turns over as a single unit in yeast is shown by the parallel loss of cytochrome oxidase and adenosine triphosphatase during sporulation (Betz & Weisner, 1976). During hypertrophy of the rat heart, after aortic constriction, similar changes are seen in the low-temperature spectra of the inner-membrane cytochromes $a+a_3$, b and c (Albin *et al.*,

1973). These authors concluded that changes in the rate of synthesis and degradation of cytochrome c were similar to that of other components of the inner membrane. Rabinowitz (1973) has estimated the turnover of the haem groups of cytochrome c and cytochrome oxidase and has shown that they possess similar half-lives.

The isotope ratios for protein subunits in a preparation of partially purified cytochrome oxidase show some heterogeneity (Fig. 1e), which may indicate differential turnover of the polypeptide components of the enzyme complex. However, this suggestion must be qualified by adding that heterogeneous turnover of contaminating proteins could produce the observed effects. Measurement of isotope ratios for the subunits of the immunisolated cytochrome oxidase was thwarted by very low incorporation of the ^{14}C label, but easily measurable incorporation of [^3H]arginine was observed (Fig. 2). The highest incorporation is into protein subunits with mol.wts. greater than 20000, which would include subunits that are thought to be synthesized on mitochondrial ribosomes (Yatscoff & Freeman, 1977).

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