The reconstitution of L-3-glycerophosphate–cytochrome *c* oxidoreductase from L-3-glycerophosphate dehydrogenase, ubiquinone-10 and ubiquinol–cytochrome *c* oxidoreductase

Ian R. COTTINGHAM and C. Ian RAGAN*

Department of Biochemistry, University of Southampton, Southampton SO9 3TU, U.K.

(Received 16 January 1980/Accepted 8 May 1980)

Purified L-3-glycerophosphate dehydrogenase from pig brain mitochondria interacts with ubiquinone-10 and ubiquinol–cytochrome *c* oxidoreductase (Complex III) from bovine heart mitochondria to reconstitute antimycin-sensitive L-3-glycerophosphate–cytochrome *c* oxidoreductase. This activity is completely dependent on the two enzymes and largely dependent on ubiquinone-10. Reconstitution requires that the two enzymes should be simultaneously present in the same membranous aggregate produced by removal of detergent from the enzymes. Reconstitution by removing detergent by dialysis or dilution is inefficient because of self-aggregation of the dehydrogenase. Highly efficient reconstitution can be achieved if the enzymes are co-precipitated by addition of ethanol. The rate with reconstituted enzyme approaches that expected from the turnover of the dehydrogenase with ubiquinone-1 as acceptor. The behaviour of the reconstituted system shows some of the characteristics expected for a stoichiometric association of one molecule of dehydrogenase with one molecule of Complex III. On raising the phospholipid/protein ratio, the dehydrogenase and Complex III appear to operate as independent enzymes acting in sequence. These effects are very similar to those observed for the interaction of NADH dehydrogenase and Complex III and are explained in terms of the model proposed by Heron, Ragan & Trumpower [(1978) *Biochem. J.* **174**, 791–800].

The lipoprotein complexes of the inner mitochondrial membrane (Hatefi, 1963) can be re-assembled to reconstitute electron transport from substrates to oxygen. For reconstitution to occur, the constituent Complexes must be present in the same membrane aggregate. This has been achieved by mixing of the detergent-solubilized enzymes, followed by removal of detergent either by dialysis or by dilution (Hatefi *et al.*, 1962; Fowler & Richardson, 1963). In a more detailed study of the association of Complex I (NADH–ubiquinone oxidoreductase) with Complex III (ubiquinol–cytochrome *c* oxidoreductase), two types of behaviour were observed. With the Complexes as isolated, stoichiometric association of the two enzymes took place such that NADH–cytochrome *c* oxidoreductase activity was nearly maximal when the molar ratio of Complex I to Complex III was 1:1 (Ragan & Heron, 1978). In the presence of a higher phospholipid/protein ratio, stoichiometric

association no longer appeared to take place and the reconstituted activity was a function of the potential maximum turnover numbers of the constituent enzymes irrespective of the molar ratio (Heron *et al.*, 1978). The reconstituted activity could be accurately accounted for by the 'Q-pool' model of Kröger & Klingenberg (1973*a,b*). Further studies, including the effects of lipid-phase transitions on the reconstituted system (Heron *et al.*, 1979), led to the proposal that, in the mitochondrial membrane, electron transfer takes place preferentially through stoichiometric Complex I–Complex III units which rapidly dissociate and reassociate at random. The rapid relative mobility of the Complexes in the dissociated state is the basis of 'Q-pool' behaviour.

The present paper describes experiments on the reconstitution of L-3-glycerophosphate dehydrogenase (Cottingham & Ragan, 1980) with Complex III. The purpose of the work was firstly to establish whether the purified dehydrogenase was competent to donate electrons to ubiquinone-10 and the cytochrome chain and secondly to investigate the

* To whom reprint requests should be addressed.

nature of the interaction by similar methods to those used for the Complex I–Complex III system. The majority of the experiments described below were performed with the partially purified L-3-glycerophosphate dehydrogenase (so-called ‘cholate-enzyme’; Cottingham & Ragan, 1980), since the presence of Triton X-100 in the pure enzyme was potentially deleterious to reconstitution.

Materials and methods

Enzyme preparations

L-3-Glycerophosphate dehydrogenase from pig brain (Cottingham & Ragan, 1980), Complex I from bovine heart (Hatefi & Rieske, 1967) and Complex III from bovine heart (Rieske, 1967*b*) were purified as described in the references. Molar concentrations of enzyme solutions were based on non-covalently bound FAD or FMN (for L-3-glycerophosphate dehydrogenase and Complex I respectively) and the cytochrome c_1 content (for Complex III) (Rieske, 1967*b*).

Chemicals

Sources of ubiquinone analogues and phospholipids are described in the preceding paper (Cottingham & Ragan, 1980). 6-Decyl-2,3-dimethoxy-5-methyl-1,4-benzoquinol was prepared by reduction of the benzoquinone as described by Rieske (1967*a*).

Assays

L-3-Glycerophosphate dehydrogenase was assayed with ubiquinone-1 as acceptor (Cottingham & Ragan, 1980). NADH–cytochrome c oxidoreductase was measured as described by Ragan & Heron (1978). L-3-Glycerophosphate–cytochrome c oxidoreductase was measured by the increase in A_{550} of a solution containing 20 μ mol of potassium phosphate, pH 8.0, 50 μ mol of DL-3-glycerophosphate, 50 nmol of cytochrome c , 10 nmol of EDTA, 10 nmol of NaN_3 and 5–50 μ g of enzyme protein in 1 ml final volume at 25°C. The oxidation of 6-decyl-2,3-dimethoxy-1,4-benzoquinol by cytochrome c was measured under the same conditions, except that 60 nmol of benzoquinol replaced the DL-3-glycerophosphate and the amount of enzyme was decreased to about 2 μ g of Complex-III protein. Non-covalently bound FAD and FMN were measured by the method of Faeder & Siegel (1973). Assays of protein, phospholipid P and ubiquinone-10 were as described in the preceding paper (Cottingham & Ragan, 1980). Sucrose concentrations in density-gradient experiments were measured with a refractometer.

Reconstitution by dilution

L-3-Glycerophosphate dehydrogenase was dissolved in 30 mM-potassium phosphate, pH 7.6, and 0.5% (w/v) cholate, to give concentrations of at least 10 mg of protein/ml. Complex III was dissolved in 0.67 M-sucrose/50 mM-Tris/HCl, pH 8.0, to similar concentrations without further addition of detergent. The solutions were mixed and diluted directly into the assay mixture for measurement of L-3-glycerophosphate–cytochrome c oxidoreductase activity unless otherwise stated. Ubiquinone-10 was dissolved in ethanol by warming to 50°C to give a 5 mM stock solution that was subsequently kept at –20°C. As required, the ubiquinone-10 was redissolved by warming and the solution diluted with warm (40°C) ethanol as necessary. Where present, ubiquinone-10 was added to the concentrated mixture of enzymes so that the final ethanol concentration did not exceed 5% (w/v).

Reconstitution by ethanol precipitation

Concentrated solutions of the two enzymes at 4°C were continuously agitated with a vortex mixer while cold ethanol or a solution of ubiquinone-10 in ethanol was rapidly injected with a micro-syringe until the final ethanol concentration was 45% (v/v). After further mixing for 10 s, the samples were diluted with 0.67 M-sucrose/50 mM-Tris/HCl, pH 8.0, at 4°C so that the final ethanol concentration was less than 5% (v/v). The activity of these diluted samples was stable for at least 3 h at 4°C. Where phospholipid was included in the reconstitution, stock solutions of soya-bean phosphatidylcholine were prepared by ultrasonic irradiation of the lipid in water to give dispersions containing 5 mM-phospholipid P (Heron *et al.*, 1978). Suitable volumes of these solutions were added to the mixture of enzymes before precipitation with ethanol.

Pure L-3-glycerophosphate dehydrogenase could also be reconstituted by the above procedure after removal of Triton X-100 by Amberlite XAD-2. The enzyme [0.4 mg of protein/ml of 30 mM-potassium phosphate, pH 7.6, and 0.1% (w/v) Triton X-100] was incubated with Amberlite XAD-2 which had been washed extensively with water. Per ml of enzyme solution, 100 mg of moist Amberlite XAD-2 was added. After 1 h at 4°C, the enzyme solution was decanted from the beads and 10% (w/v) potassium cholate added to a final concentration of 0.5% (w/v).

Antimycin inhibition

Antimycin was dissolved in ethanol at several concentrations so that the required amounts could be added to the enzyme mixture in the same volume of ethanol (usually an amount to give a final concentration of 5%, v/v). After 1 h at 4°C,

reconstitution was performed as described in the previous section, taking account of the ethanol already present in the mixture.

Theoretical analysis of 'Q-pool' behaviour

Kröger & Klingenberg (1973a) found that the rate of reduction of endogenous ubiquinone and the rate of oxidation of endogenous ubiquinol by submitochondrial particles were first-order with respect to the concentrations of ubiquinone and ubiquinol respectively. Thus V , the overall rate of substrate oxidation by oxygen, is related to Q_{ox} , the concentration of ubiquinone, or Q_{red} , the concentration of ubiquinol, by the expressions:

$$V = V_{red} \cdot \frac{Q_{ox}}{Q} = V_{ox} \cdot \frac{Q_{red}}{Q}$$

where $Q = Q_{ox} + Q_{red}$, V_{red} is the maximum rate of the ubiquinone-reducing system (i.e. when $Q_{ox} = Q$) and V_{ox} is the maximum rate of the ubiquinol-oxidizing system (i.e. when $Q_{red} = Q$). These expressions may be combined to give

$$V = \frac{V_{ox} \cdot V_{red}}{V_{ox} + V_{red}} \quad (1)$$

Heron *et al.* (1978) adapted this equation to the titration of Complex I with Complex III and derived the following expression, which of course is equally suited to the titration of L-3-glycerophosphate dehydrogenase and Complex III:

$$V = \frac{V_1 \cdot N}{\frac{x_3 V_1}{x_1 V_3} + N} \quad (2)$$

where: V is the L-3-glycerophosphate-cytochrome *c* oxidoreductase activity/mg of L-3-glycerophosphate dehydrogenase; V_1 is the maximum value of this rate (e.g. at saturating concentration of Complex III), corresponding to V_{red} in eqn. (1); V_3 is the maximum rate of electron transfer through Complex III expressed as the cytochrome *c* reductase activity/mg of Complex III, and corresponding to V_{ox} in eqn. (1); N is the molar ratio of Complex III to dehydrogenase; x_1 is the nmol of FAD/mg of dehydrogenase; and x_3 is the nmol of cytochrome *c*₁/mg of Complex III.

This expression shows that V varies hyperbolically with N . The curve has a V_{max} value equal to V_1 and a K_m value equal to $x_3 V_1 / x_1 V_3$.

Eqn. (2) can be rearranged to express the rate of L-3-glycerophosphate-cytochrome *c* oxidoreductase activity/mg of Complex III as a function of the molar ratio of dehydrogenase to Complex III. Thus, if V is the rate/mg of dehydrogenase, V/x_1 is the rate/nmol of dehydrogenase, $V/x_1 N$ is the rate/nmol

of Complex III and $x_3 V / x_1 N$ is the rate/mg of Complex III. Eqn. (2) then becomes

$$\frac{x_3 V}{x_1 N} = \frac{V_1 N \frac{x_3}{x_1 N}}{\frac{x_3 V_1}{x_1 V_3} + N} = \frac{V_3 \cdot \frac{1}{N}}{\frac{x_1 V_3}{x_3 V_1} + \frac{1}{N}} \quad (3)$$

Eqn. (3) shows that the curve relating rate per mg of Complex III to the molar ratio of dehydrogenase to Complex III ($1/N$) is also hyperbolic. The V_{max} is equal to V_3 and the K_m is equal to $x_1 V_3 / x_3 V_1$. Thus V_1 and V_3 can be evaluated from two reciprocal plots and be used to calculate the K_m values, one of which should be the reciprocal of the other.

The approach used here dispenses with the need to determine V_3 with the use of artificial donors (Kröger & Klingenberg, 1973a; Heron *et al.*, 1978) and therefore is less likely to be prone to systematic errors.

Results from titration experiments were fitted to a hyperbolic curve by using an unweighted least-squares regression analysis (Wilkinson, 1961), thus giving values for K_m and V_{max} .

Theoretical analysis of antimycin-inhibition curves

The inhibition of ubiquinol oxidation by antimycin is linear with antimycin concentration (Kröger & Klingenberg, 1973b) and this is verified below. Thus V_3 is decreased in the presence of antimycin to $V_3(1-n)$, where n is the molar ratio of antimycin to Complex III. Eqn. (2) now becomes

$$V = \frac{V_1 \cdot N}{\frac{x_3 V_1}{x_1 V_3(1-n)} + N} = \frac{V_1 \cdot N}{\frac{K_m}{1-n} + N} \quad (4)$$

where K_m is that measured from a titration of uninhibited Complex III with dehydrogenase as described above.

The rate in the presence of antimycin as a fraction of the original rate (r) is then obtained from eqns. (2) and (4):

$$r = \frac{K_m + N}{\left(\frac{K_m}{1-n}\right) + N} = \frac{(K_m + N)(1-n)}{K_m + N(1-n)} \quad (5)$$

Thus the degree of inhibition is a function of both N and n . K_m is a constant and can be determined by separate experiments, and therefore the antimycin-inhibition curve can be predicted from eqn. (5).

Results

Reconstitution of L-3-glycerophosphate-cytochrome *c* oxidoreductase by dilution

Following the procedure of Hatefi *et al.* (1962), initial attempts to reconstitute this activity involved

Table 1. *Reconstitution of L-3-glycerophosphate-cytochrome c oxidoreductase activity by dilution*

L-3-Glycerophosphate dehydrogenase (200 μ g of protein containing 0.14 nmol of FAD) was mixed with Complex III to give the indicated molar ratios. In some experiments ubiquinone-10 was also added. Samples were diluted directly into the assay medium for L-3-glycerophosphate-cytochrome c oxidoreductase activity as described in the Materials and methods section.

Complex III (mol/mol of dehydrogenase)	Ubiquinone-10 (nmol)	L-3-Glycerophosphate-cytochrome c oxidoreductase (μ mol of cytochrome c/ min per mg of dehydrogenase)
0	10	<0.001
23	10	0.14
57	10	0.51
115	10	1.14
115	5	0.81
115	0	0.40

mixing L-3-glycerophosphate dehydrogenase [dissolved in 0.5% (w/v) cholate] with Complex III and diluting the mixture to remove detergent. Table 1 shows that this was successful and that the reconstituted activity was stimulated by addition of ubiquinone-10. Neither constituent enzyme alone showed any L-3-glycerophosphate-cytochrome c oxidoreductase activity. The highest rate achieved in the experiment of Table 1 (1.14 μ mol of cytochrome c/min per mg of dehydrogenase protein) was 70% of that expected from the activity of the dehydrogenase towards ubiquinone-1 (0.81 μ mol of ubiquinone-1/min per mg of dehydrogenase protein or 1.62 μ equiv. of electrons transferred/min per mg of dehydrogenase protein). Thus the partially purified dehydrogenase was able to transfer electrons to the cytochromes at rates comparable with the turnover with artificial electron acceptors such as ubiquinone-1. However, the maximum reconstituted activity in general exceeded the activity of the dehydrogenase with dichlorophenol-indophenol as acceptor (Cottingham & Ragan, 1980), which implies that this acceptor underestimates the potential turnover under physiological conditions.

In Table 1, the relative amounts of L-3-glycerophosphate dehydrogenase and Complex III have been expressed as a molar ratio. Although it is reasonably safe to base the Complex-III molarity on the cytochrome c_1 content (Rieske, 1976), the molarity of the dehydrogenase is harder to establish. We have used the FAD content as a measure of this, but, since the enzyme is impure and prone to loss of flavin, there is a degree of uncertainty about the accuracy of such values. Whether based on the molar ratio of Complex III to dehydrogenase or on the relative activities of the two enzymes, the amounts of Complex III required to produce maximal reconstituted activities are surprisingly high (Table 1). The poor rates at lower molar ratios were not substantially increased by addition of ubiquinone-10, which suggested that some other factor was rate-limiting. The likely explanation was that co-aggregation of dehydrogenase and Complex III was a rather inefficient process, except in the

Fig. 1. *Reconstitution by dilution*

L-3-Glycerophosphate dehydrogenase (15.2 μ M) was mixed with various volumes of Complex III (49 μ M) or 0.67 M-sucrose/50 mM-Tris/HCl, pH 8.0 at 0°C, to give the indicated molar ratios and a final concentration of 1.52 μ M-dehydrogenase. Samples (10 μ l) were then (a) assayed immediately for L-3-glycerophosphate-cytochrome c oxidoreductase or (b) diluted 3-fold with sucrose/Tris buffer, incubated for 30 min at 0°C and then assayed.

presence of a large excess of Complex III. The reasons for the inefficiency are indicated by the experiment of Fig. 1, which shows the reconstituted activity as a function of Complex-III concentration under two different sets of conditions. Curve (a) shows the rate obtained when the detergent-solubilized dehydrogenase and Complex-III mixtures were diluted directly into the assay medium (200-fold dilution). Curve (b) shows the rates when the enzyme mixtures were firstly diluted 3-fold, incubated for 30 min at 0°C, and then diluted into the assay medium [a total dilution of 200-fold, as for curve (a)]. The triphasic curve (b) can be explained as follows. At low concentrations of Complex III the two-step dilution process produces the same rates as the single-step dilution. At higher concentrations of Complex III, the increased detergent concentration introduced with the Complex III is sufficient to keep

this enzyme in solution after 3-fold dilution, whereas the less-soluble dehydrogenase self-aggregates. Thus, when the mixture is diluted into the assay, the Complex III and dehydrogenase are not efficiently co-aggregated and activity is low. At even higher concentrations of Complex III (and detergent), neither enzyme precipitates to a great extent as a result of 3-fold dilution, and the rates after dilution into the assay mixture approach those obtained with a single 200-fold dilution of the mixture.

Most efficient reconstitution therefore occurs when the samples are diluted to the greatest possible extent in one stage. This overcomes to some extent the tendency of the less-soluble dehydrogenase to self-aggregate rather than associate with the more-soluble Complex III. This is also overcome by the use of very high concentrations of Complex III, thus explaining the very high molar ratios needed to give reasonable rates of L-3-glycerophosphate-cytochrome *c* oxidoreductase activity. No improvement was found if the enzymes were precipitated by dialysing away the detergent. To achieve efficient reconstitution at low molar ratios of Complex III to dehydrogenase, it was found necessary to use organic solvents to co-precipitate the enzymes.

Reconstitution of L-3-glycerophosphate-cytochrome c oxidoreductase by ethanol precipitation

As shown in Fig. 2, the reconstituted activity was greatly improved by addition of ethanol to the mixture of solubilized enzymes before dilution. The optimal concentration was about 45% (w/v), a concentration that caused the solution to become immediately turbid. Thus the increased activity could be explained by greater co-precipitation of the two enzymes. The decrease in activity at higher ethanol concentrations was due to inactivation of the constituent enzymes. Surprisingly, the reconstituted activity was not decreased by extending the period of exposure to 43.5% (w/v) ethanol at 4°C from 10 s to

5 min. After a further 5 min, the activity had only declined by 27%. However, in the presence of 51.8% (w/v) ethanol, the reconstituted activity was unstable and decreased with a half-time of approx 1 min. At even higher concentrations of ethanol, inactivation occurred too rapidly to be prevented by subsequent dilution of the ethanol (Fig. 2). Complex III proved to be the more sensitive of the two constituent enzymes to inactivation by ethanol. Thus, in the experiment of Table 2, no loss of L-3-glycerophosphate dehydrogenase activity was found whether ethanol precipitation occurred in the

Fig. 2. *Effects of ethanol on reconstitution*
Mixtures of L-3-glycerophosphate dehydrogenase and Complex III in a 1.43:1 molar ratio were mixed with the indicated concentrations of ethanol as described in the Materials and methods section. After 10 s exposure to ethanol, the samples were diluted and assayed for L-3-glycerophosphate-cytochrome *c* oxidoreductase as described in the Materials and methods section.

Table 2. *Effects of ethanol precipitation on enzyme activities*

Where indicated, L-3-glycerophosphate dehydrogenase (0.08 nmol) and Complex III (0.055 nmol) were reconstituted by ethanol precipitation in the presence of 5 nmol of ubiquinone-10 as described in the Materials and methods section and assayed for L-3-glycerophosphate-ubiquinone-1 oxidoreductase, L-3-glycerophosphate-cytochrome *c* oxidoreductase or 6-decyl-2,3-dimethoxy-5-methyl-1,4-benzoquinol-cytochrome *c* oxidoreductase activities. Abbreviation: n.d., not done.

Enzyme present	Ethanol precipitation	Rate of oxidation of L-3-glycerophosphate with indicated acceptor (μmol of acceptor/min per mg of dehydrogenase)		Benzoquinol-cytochrome <i>c</i> oxidoreductase (μmol of cytochrome <i>c</i> /min per mg of Complex III)
		Ubiquinone-1	Cytochrome <i>c</i>	
Dehydrogenase	—	0.55	0.00	n.d.
Dehydrogenase	+	0.61	0.00	n.d.
Complex III	—	n.d.	0.00	23.4
Complex III	+	n.d.	0.00	7.0
Complex III and dehydrogenase	+	0.62	0.43	19.5

presence or absence of Complex III. On the other hand, Complex III was substantially inactivated when precipitated with 45% (w/v) ethanol in the absence of the dehydrogenase, but was protected from inactivation when the dehydrogenase was present. This stabilization effect might be indicative of a specific association between dehydrogenase and Complex III. It is also possible, though, that the effect might be non-specific and be due only to the higher total protein concentration. Table 2 also shows that ethanol-precipitated dehydrogenase or Complex III totally lacked L-3-glycerophosphate-cytochrome *c* oxidoreductase activity.

The ability of ethanol to stimulate activity was shared by other lower alcohols such as methanol and propanol, but no systematic study of these solvents was made.

Dependence of reconstituted activity on ubiquinone-10

Once the dehydrogenase and Complex III had been co-precipitated with ethanol, the rate of cytochrome *c* reductase activity was limited by ubiquinone-10 rather than by the efficiency of association of the two enzymes. As shown in Table 3, at two molar ratios of Complex III to dehydrogenase, the stimulation by ubiquinone-10 was 8–9-fold, and the rates were almost completely sensitive to antimycin. Table 3 also shows that, even at low molar ratios of Complex III to dehydrogenase, substantial activity could be obtained, in contrast with the results of Fig. 1, for example.

Fig. 3 shows the dependence of reconstituted activity on ubiquinone-10. Maximal activity was obtained at approx. 30 mol of ubiquinone-10/mol of Complex III, though it is possible that not all the ubiquinone-10 was incorporated into the membrane aggregate. The small activity in the absence of added ubiquinone-10 can be attributed to the endogen-

ous ubiquinone-10 of both the dehydrogenase (0.51 nmol/mg of protein) and the Complex III (2.5 nmol/mg of protein), which together added up to 1.6 mol of ubiquinone-10/mol of Complex III. Since this amount supported 19% of the maximal activity, it seems likely that not all the added ubiquinone-10 was incorporated.

Physical association of the constituent enzymes

Ethanol-precipitated Complex III or ethanol-precipitated L-3-glycerophosphate dehydrogenase sedimented as single bands of activity on sucrose density gradients with densities equal to those of 44% (w/v) and 51% (w/v) sucrose respectively. If the ethanol-precipitated enzymes were mixed before centri-

Fig. 3. *Dependence of reconstituted activity on ubiquinone-10*

Mixtures containing 0.7 mol of Complex III/mol of L-3-glycerophosphate dehydrogenase were reconstituted by addition of an ethanolic solution of ubiquinone-10 (10 μ M–5 mM) to give the indicated molar ratios of ubiquinone-10 to Complex III and a final ethanol concentration of 45% (v/v). Samples were then diluted 15-fold and assayed for L-3-glycerophosphate-cytochrome *c* oxidoreductase.

Table 3. *Effect of ubiquinone-10 on the reconstituted activity*

L-3-Glycerophosphate dehydrogenase was reconstituted with Complex III in the indicated molar ratios by ethanol precipitation. In some experiments ubiquinone-10 was included before precipitation. Samples were assayed for L-3-glycerophosphate-cytochrome *c* oxidoreductase in the presence and absence of 1 μ g of antimycin as described in the Materials and methods section.

Concn. of Complex III (mol/mol of dehydrogenase)	Concn. of ubiquinone-10 (mol/mol of dehydrogenase)	L-3-Glycerophosphate- cytochrome <i>c</i> oxidoreductase (μ mol of cytochrome <i>c</i> /min per mg of dehydrogenase)
0.7	0	0.084
0.7	310	0.66
0.7	620	0.50
0.7	310	0.01 (+ antimycin)
2.8	0	0.16
2.8	330	1.55
2.8	660	1.39
2.8	330	0.08 (+ antimycin)

fugation or if the enzymes were co-precipitated with ethanol, a single band of intermediate density [equal to 46% (w/v) sucrose] was formed, which contained both enzymes. However, the L-3-glycerophosphate-cytochrome *c* oxidoreductase activity was approx. 10-fold higher in the band formed from co-precipitated enzymes. Although the formation of a band of intermediate density clearly shows that co-aggregation of the enzymes has taken place, this is not in itself a sufficient condition for the appearance of L-3-glycerophosphate-cytochrome *c* oxidoreductase.

Dependence of L-3-glycerophosphate-cytochrome c oxidoreductase activity on the molar ratio of the constituent enzymes

Fig. 4 shows the dependence of reconstituted activity (per mg of Complex-III protein) on the molar ratio of dehydrogenase to Complex III. Reconstitution was performed under optimal conditions of ethanol and ubiquinone-10 concentrations. Despite the high turnover of Complex III in

the ubiquinol-cytochrome *c* oxidoreductase assay, the maximum rate did not exceed 1 μ mol of cytochrome *c*/min per mg of Complex-III protein. Thus Complex III does not appear to be able to reach its potential turnover in the reconstituted system. Maximal activity was reached at very close to 1 mol of dehydrogenase/mol of Complex III. These results suggest that the enzymes form a stoichiometric unit containing one molecule of each. The turnover of such a unit would therefore be limited by the slowest component (i.e. the dehydrogenase), therefore explaining the low turnover of the Complex III. Whether the stoichiometry is truly 1 mol of dehydrogenase/mol of Complex III depends on whether the preparation of 'cholate-enzyme' is free from other FAD-containing proteins and whether enzyme that might have lost its FAD is capable of binding to Complex III. In view of these uncertainties, further experiments were done which tested the idea of stoichiometric association without requiring a knowledge of the actual numbers of molecules involved. One prediction is that decreasing the turnover of the dehydrogenase should decrease the turnover of the Complex III to the same extent. In Fig. 4, activities were also measured with a subsaturating concentration of DL-3-glycerophosphate (4 mM). The ubiquinone-1 reductase activity was decreased to 30% of that measured with 50 mM

Fig. 4. *Dependence of reconstituted activity on the molar ratio of dehydrogenase to Complex III*

Mixtures containing a fixed amount of L-3-glycerophosphate dehydrogenase and Complex III to give the indicated molar ratios were reconstituted by ethanol precipitation in the presence of 50–100 nmol of ubiquinone-10/mol of Complex III. The actual amount was allowed to vary between these limits to avoid the need to make up different concentrations of ubiquinone-10 in ethanol for each molar ratio of enzymes. Fig. 3 shows that the reconstituted rate was not very sensitive to changes in ubiquinone-10 concentration between 30 and 100 nmol/mol of Complex III. Reconstituted samples were diluted and assayed for L-3-glycerophosphate-cytochrome *c* oxidoreductase in the presence of (●) 50 mM- or (▲) 4 mM-DL-3-glycerophosphate.

Fig. 5. *Antimycin inhibition as a function of the molar ratio of Complex III to dehydrogenase*

Reconstitution of mixtures containing (●) 3.5 mol or (▲) 0.185 mol of Complex III per mol of dehydrogenase was performed in the presence of 50 nmol of ubiquinone-10/mol of Complex III and the indicated concentrations of antimycin as described in the Materials and methods section. Samples were diluted and assayed for L-3-glycerophosphate-cytochrome *c* oxidoreductase. Rates were calculated per mg of dehydrogenase protein and expressed as the percentage of the rate in the absence of inhibitor.

substrate. Rather than the reconstituted activity reaching the same maximum value with 3.3-fold more dehydrogenase (expected for independent enzymes operating in sequence), the curve obtained approximated more closely to that expected for stoichiometric interaction (maximum rate still reached at a 1:1 molar ratio, but decreased to 30% of the original). The rate did increase at molar ratios greater than 1, and the decrease in maximum rate was only 50%. Thus the actual behaviour was somewhere between the two theoretical extremes.

A further prediction of stoichiometric interaction is that the percentage inhibition of the reconstituted activity by a particular antimycin/Complex-III ratio should be independent of the Complex-III/dehydrogenase ratio (Ragan & Heron, 1978). Such an experiment is shown in Fig. 5. In practice, inhibition by antimycin was less in the presence of a high Complex-III/dehydrogenase ratio, as expected if the enzymes were not fully associated into stoichiometric units. However, the antimycin-titration curves did not fit the lines predicted for 'Q-pool' behaviour (i.e. independent enzymes), as described in more detail in the next section.

Restoration of 'Q-pool' behaviour to the reconstituted system

The association of Complex I with Complex III could be changed from a stoichiometric interaction to one which exactly fitted the equations of 'Q-pool' behaviour by increasing the phospholipid/protein ratio (Heron *et al.*, 1978, 1979). The following experiments show that the same phenomenon occurred with the L-3-glycerophosphate dehydrogenase-Complex III system. Fig. 6 shows the effects on reconstituted activity of including phospholipid before ethanol precipitation. The stimulation of activity was greater at a low Complex-III/dehydrogenase ratio than at a higher ratio, showing that phospholipid had altered the shape of titration curves such as those of Fig. 4. A similar experiment to that of Fig. 4 was therefore performed in the presence of phospholipid (Fig. 7). Both the shape of the curve and the maximum rates were altered. In particular, the maximum rate/mg of Complex-III protein was some 10-fold higher in the presence of phospholipid, and moreover it was much the same at either L-3-glycerophosphate concentration. The inset shows that the curves were hyperbolic, and the $V_{\max}$ and K_m values for each are summarized in Table 4. A replot of the data of Fig. 7, expressing the rates/mg of dehydrogenase protein as a function of the molar ratio of Complex III to dehydrogenase, also gave hyperbolic curves (Fig. 8 and inset), from which the other kinetic parameters of Table 4 were obtained. Table 4 also presents theoretical calculations of K_m determined from the $V_{\max}$ values

Fig. 6. *Effect of phospholipid on reconstitution*
L-3-Glycerophosphate dehydrogenase was mixed with Complex III to give molar ratios of (●) 0.224 mol or (▲) 2.02 mol of Complex III/mol of dehydrogenase. Samples containing 100 μg of dehydrogenase protein were reconstituted by ethanol precipitation in the presence of the indicated concentrations of soya-bean phosphatidylcholine and 50 nmol of ubiquinone-10/mol of Complex III as described in the Materials and methods section. After dilution, the samples were assayed for L-3-glycerophosphate-cytochrome *c* oxidoreductase activity.

Fig. 7. *Dependence of reconstituted activity on the molar ratio of dehydrogenase to Complex III in the presence of additional phospholipid*

Reconstitution was performed similarly to the experiment of Fig. 4, except that soya-bean phosphatidylcholine (200 nmol/mg of dehydrogenase protein) was present in each sample. Samples were diluted and assayed for L-3-glycerophosphate-cytochrome *c* oxidoreductase (per mg of Complex-III protein) in the presence of (●) 50 mM or (▲) 6 mM DL-3-glycerophosphate. The inset shows a double-reciprocal plot of the data. For clarity of drawing, some data points have been omitted from the Figure. Calculations of K_m and $V_{\max}$ were performed with 12 pairs of measurements.

Table 4. Comparison of theoretical and experimental K_m values

The V_{max} and K_m values from Fig. 7 are shown as V_3 and K_m (dehydrogenase). The corresponding values from Fig. 8 are shown as V_1 and K_m (Complex III). Theoretical K_m values were calculated from V_1 , V_3 , x_1 ($= 0.47$ nmol of FAD/mg of dehydrogenase) and x_3 ($= 3.7$ nmol/mg of Complex III) as described in the Materials and methods section. All values are means $\pm$ S.E.M. ($n = 12$).

	Kinetic parameters determined with the following DL-3-glycerophosphate concentrations	
	50 mM	6 mM
V_3	10.8 ± 0.7	9.3 ± 0.2
V_1	1.91 ± 0.10	0.76 ± 0.02
K_m (dehydrogenase)	0.76 ± 0.13	1.93 ± 0.14
K_m (Complex III)	1.31 ± 0.25	0.72 ± 0.08
Theoretical K_m (dehydrogenase)	0.76 ± 0.09	1.65 ± 0.07
Theoretical K_m (Complex III)	1.32 ± 0.25	0.61 ± 0.03

Fig. 8. Dependence of reconstituted activity on the molar ratio of Complex III to dehydrogenase in the presence of additional phospholipid

The data of Fig. 7 were recalculated to give rates/mg of dehydrogenase protein as a function of the Complex-III/dehydrogenase ratio. The inset is a double-reciprocal plot of the data. Again, some points were omitted for clarity, but K_m and V_{max} calculations were performed with all 12 pairs of measurements.

as described in the Materials and methods section. The agreement with the experimental values was very good, showing that ‘Q-pool’ behaviour had been restored. The main difference between the behaviour with and without phospholipid lies in the turnover of Complex III. When the enzymes are stoichiometrically associated, the Complex-III turnover is necessarily limited to that of the dehydrogenase. During ‘Q-pool’ behaviour, the Complex-III turnover in the reconstituted system can approach that of the isolated enzyme at high enough dehydrogenase/Complex-III ratios.

Fig. 9. Inhibition of benzoquinol-cytochrome c oxidoreductase by antimycin

Reconstitution of mixtures containing (●) 3.5 mol or (▲) 0.185 mol of Complex III/mol of dehydrogenase was performed in the presence of 50 nmol of ubiquinone-10/mol of Complex III, 200 nmol of phosphatidylcholine/mg of dehydrogenase protein, and the indicated amounts of antimycin. Samples were diluted and assayed for 6-decyl-2,3-dimethoxy-5-methyl-1,4-benzoquinol-cytochrome c oxidoreductase activity as described in the Materials and methods section.

Antimycin titrations of the reconstituted system could also be predicted by the equations of ‘Q-pool’ behaviour. As shown in Fig. 9, the quinol-cytochrome c oxidoreductase activity of the reconstituted system was inhibited by antimycin in a linear fashion and the curves were identical at two widely different Complex-III/dehydrogenase ratios. In contrast, the antimycin-inhibition curves for L-3-glycerophosphate-cytochrome c oxidoreductase activity were very much dependent on the Complex-III/dehydrogenase ratio (Fig. 10). Theoretical

curves were calculated as described in the Materials and methods section by using a K_m value for Complex III of 0.25 mol/mol of dehydrogenase. The fit is excellent up to an antimycin concentration of 0.9 mol/mol of Complex III. Deviation from the theoretical line can be explained by an appreciable dissociation constant for antimycin (Kröger & Klingenberg, 1973b). Thus 100% inhibition is not obtained at 1 mol of antimycin/mol of Complex III, as shown by Fig. 9. The residual, uninhibited, activity makes a much greater contribution to the

Fig. 10. Antimycin inhibition as a function of the molar ratio of Complex III to dehydrogenase in the presence of phospholipid

Conditions were identical with those of Fig. 9, except that samples were assayed for L-3-glycerophosphate-cytochrome *c* oxidoreductase activity. Activities are expressed as a percentage of the rates in the absence of antimycin. The lines are calculated as described in the Materials and methods section and in the text.

overall L-3-glycerophosphate-cytochrome *c* oxidoreductase activity when the Complex-III/dehydrogenase ratio is high. The curves of Fig. 10 are quite different from those of Fig. 5, in which phospholipid was omitted from the reconstitution.

Although we have chosen to express concentrations of dehydrogenase and Complex III in molarity for comparison of results with the predictions of stoichiometric association, fitting the data to the 'Q-pool' equations does not in fact require a knowledge of the molar concentration of enzymes. The 'Q-pool' equations could as well have been developed with activities expressed throughout per mg of protein. Thus the excellent agreement between experiment and theory does not depend on the rather dubious values for the molarity of the dehydrogenase.

Reconstitution with purified L-3-glycerophosphate dehydrogenase

Efficient reconstitution with the pure enzyme would establish that no polypeptides other than that of 75 000 mol.wt. were required for transfer of electrons to the cytochrome chain. The presence of Triton X-100 was extremely detrimental to the reconstitution. In one experiment, 1% (w/v) Triton X-100 was added to the mixture of Complex III and dehydrogenase before ethanol precipitation. The reconstituted activity was decreased by 85%, although the Triton concentration in the assay was only 0.0016% (w/v). To avoid this problem, Triton X-100 was removed from the purified enzyme by incubation with Amberlite XAD-2. Cholate was then added to a concentration of 0.5% (w/v) and reconstitution with Complex III was performed under conditions chosen to optimize the activity per mg of dehydrogenase (i.e. phospholipid, ubiquinone-10 and excess of Complex III). A preparation of the 'cholate-enzyme' was also treated with Amber-

Table 5. Reconstitution with purified L-3-glycerophosphate dehydrogenase

Purified L-3-glycerophosphate was treated with Amberlite XAD-2 to remove Triton X-100 as described in the Materials and methods section. Triton X-100 was added to a portion of the partially purified enzyme to a final concentration of 0.1% (w/v) and the Triton X-100 was then removed with Amberlite XAD-2 as before. The purified and partially purified enzymes were then reconstituted with Complex III (10 mol/mol of dehydrogenase), ubiquinone-10 (50 mol/mol of dehydrogenase) and soya-bean phosphatidylcholine (200 nmol/mg of dehydrogenase) by ethanol precipitation. Samples were assayed for L-3-glycerophosphate oxidation by either ubiquinone-1 or cytochrome *c*.

Enzyme sample	Rate of L-3-glycerophosphate oxidation with indicated acceptor (μ mol of acceptor/ min per mg of dehydrogenase)		Ratio cytochrome/ubiquinone
	Ubiquinone-1	Cytochrome <i>c</i>	
Partially pure	1.14	1.83	1.60
Partially pure, treated with Triton and Amberlite	0.99	1.43	1.44
Purified, treated with Amberlite	3.44	4.86	1.41

lite XAD-2 as a control. The results are shown in Table 5. Partially purified dehydrogenase gave a reconstituted rate that was 1.60-fold greater than the ubiquinone-1 reductase activity (i.e. 80% of that expected). The partially purified enzyme which had been treated with Amberlite XAD-2 had lost only a little ubiquinone-1 reductase activity, but the reconstituted rate had decreased somewhat more. Thus the Amberlite treatment had decreased the efficiency of the reconstitution to a small extent. However, the purified dehydrogenase, after Amberlite treatment, reconstituted with Complex III as efficiently as the 'cholate-enzyme'. Thus the single-subunit enzyme is fully competent in reconstitution. The small amounts of the purified enzyme that were available prevented us from studying its interaction with Complex III under other conditions. However, since the reconstituted activity was somewhat lower than expected even under optimal conditions, the pure enzyme may not have proved particularly useful.

Reconstitution of Complex I and L-3-glycerophosphate dehydrogenase with Complex III

An attractive possibility was a three-way reconstitution of Complex I, L-3-glycerophosphate dehydrogenase and Complex III, to see whether under certain circumstances the two dehydrogenases competed for Complex-III molecules. Unfortunately, ethanol co-precipitation completely inactivated Complex I (Table 6). The presence of L-3-glycerophosphate dehydrogenase stabilized the

Complex I to a small extent, but the rate of NADH-cytochrome *c* oxidoreductase was only 4% of that obtained by dilution of a Complex I-Complex III mixture. Co-aggregation by dilution caused inefficient association of L-3-glycerophosphate dehydrogenase and Complex III, and even this was totally prevented by the inclusion of Complex I. Co-aggregation by dialysis produced similar results, and no conditions were found which resulted in high rates of both NADH-cytochrome *c* oxidoreductase and L-3-glycerophosphate-cytochrome *c* oxidoreductase activities.

Discussion

The successful reconstitution of antimycin-sensitive L-3-glycerophosphate-cytochrome *c* oxidoreductase activity provides good evidence that the purified and partially purified L-3-glycerophosphate dehydrogenase described in the preceding paper (Cottingham & Ragan, 1980) had not been substantially modified during isolation. In particular, the reconstitution with the pure enzyme shows that the same polypeptide of mol.wt. 75 000 is capable of oxidizing the substrate and reducing ubiquinone-10 or the cytochromes. There is therefore no requirement for a so-called 'Q-binding protein' such as those postulated to be involved in succinate dehydrogenase, Complex I and Complex III (Yu *et al.*, 1978).

The unusual conditions required to reconstitute

Table 6. *Reconstitution of NADH- and L-3-glycerophosphate-cytochrome c oxidoreductase activities*

L-3-Glycerophosphate dehydrogenase or Complex I or both were mixed with Complex III to give molar ratios of 1 mol of dehydrogenase or 1 mol of Complex I to 5 mol of Complex III. Ubiquinone-10 (100 nmol/mol of dehydrogenase or Complex I) and soya-bean phosphatidylcholine (400 nmol/mol of dehydrogenase or Complex I) were also present throughout. Reconstitution was then performed by (a) ethanol precipitation, (b) dilution into the assay or (c) dialysis of the concentrated mixture against 500 vol. of 0.67 M-sucrose/50 mM-Tris/HCl, pH 8.0 at 4°C, for 16 h, followed by dilution into the assay. Samples were assayed for L-3-glycerophosphate- and NADH-cytochrome *c* oxidoreductase activities. Abbreviation: n.d., not done. Rates were calculated per mg of protein of the dehydrogenase being assayed, i.e. either NADH dehydrogenase (Complex I) or L-3-glycerophosphate dehydrogenase.

Mode of reconstitution	Enzymes present	Rate with indicated donor (μ mol of cytochrome <i>c</i> /min per mg of dehydrogenase or Complex I)	
		NADH	L-3-Glycerophosphate
Ethanol precipitation	Dehydrogenase, Complex III	0.00	0.238
	Complex I, Complex III	0.00	0.00
	Dehydrogenase, Complex I and Complex III	0.05	0.252
Dilution	Dehydrogenase, Complex III	n.d.	0.058
	Complex I, Complex III	1.31	n.d.
	Dehydrogenase, Complex I and Complex III	n.d.	0.00
Dialysis	Dehydrogenase, Complex III	n.d.	0.00
	Complex I, Complex III	n.d.	n.d.
	Dehydrogenase, Complex I and Complex III	1.12	0.00

L-3-glycerophosphate dehydrogenase with Complex III arise because of the tendency of the dehydrogenase to self-aggregate on removal of detergent. The processes involved in the reconstitution may be described as follows:

The first reaction is the only one that leads to reconstituted activity. The three reactions compete for the two enzymes, and the final proportion of reconstituted material will depend on the three rate constants and the initial concentrations of the two enzymes. The tendency of the dehydrogenase to self-associate means that k_{+3} is high compared with k_{+1} or k_{+2} , and k_{+2} is likely to be small compared with k_{+1} . Thus reconstitution will be inefficient unless reaction (6) is promoted by the presence of high concentrations of Complex III, as found in practice. If k_{+2} is small, then reaction (7) never competes to any extent with reaction (6).

The effect of including ethanol is clearly to increase k_{+1} (and perhaps k_{+2}) relative to k_{+3} . Indeed the titration experiments of Figs. 4 and following suggest that reaction (6) is preferred in the presence of ethanol, and that the efficiency of reconstitution can be very high. The fact that the rates of electron transfer to cytochrome *c* can, under optimal conditions, exceed 80% of the rate of electron transfer to ubiquinone-1 also suggests a high efficiency of reconstitution.

The apparently stoichiometric association of the dehydrogenase with Complex III is rather surprising, particularly as the enzymes are from different species, and bovine heart mitochondria (from which Complex III is purified) are incapable of oxidizing L-3-glycerophosphate. The evidence that the apparent stoichiometry is 1 mol of dehydrogenase/mol of Complex III rests on the rather dubious assay of dehydrogenase molarity. However, the experiments of Fig. 4, in which the turnover of Complex III was restricted by the turnover of the dehydrogenase, show that each molecule of Complex III could only receive electrons from a limited number of dehydrogenase molecules, this number being 1 on the basis of FAD content. Since the titration curves of Figs. 4 and 5 did not exactly follow the predictions of stoichiometric association, it seems that electrons are donated to Complex III most rapidly by this small number of dehydrogenase molecules and very much less rapidly by other molecules, the rate depending perhaps on the distance of the dehydrogenase from the Complex III.

Slow diffusion of ubiquinone-10 between dehydrogenase and Complex III molecules is unlikely as an explanation, since ubiquinone-10 was saturating. The results suggest therefore that electron transfer is controlled by the rates of protein diffusion in the membrane, implying some kind of association between the dehydrogenase and Complex III. The simplest kind of association would be a 1:1 interaction of the type proposed for Complex I and Complex III (Ragan & Heron, 1978). Deviations from strict stoichiometric association would be explained if the binary unit was able to dissociate and re-form with other molecules at rates comparable with, but rather slower than, the rate of overall electron transfer.

If the rate of reversible association of the dehydrogenase and Complex III was increased so that it was no longer rate-limiting for overall electron transfer, then all dehydrogenase molecules would transfer electrons to all Complex-III molecules at equal rates. This is what appears to be happening when the phospholipid/protein ratio is increased and 'Q-pool' behaviour is restored. 'Q-pool' behaviour does not require a reversible association of dehydrogenase with Complex III and was originally explained by a homogeneous, mobile pool of ubiquinone-10 molecules (Kröger & Klingenberg, 1973a). However, the same kinetic behaviour would be expected as long as the binary units associate and dissociate fast enough (Heron *et al.*, 1978). Whatever the explanation of 'Q-pool' behaviour, the reconstituted L-3-glycerophosphate-cytochrome *c* oxidoreductase activity follows the predictions of the model very closely. The advantage of the reconstituted system is that it is possible to vary the numbers of molecules of the participating enzymes as well as varying their activity by inhibitors. It is therefore possible to verify directly the proposal of Kröger & Klingenberg (1973a) that it is the total activities of the ubiquinone-reducing system and the ubiquinol-oxidizing system that determine the overall rate, and not the relative numbers of molecules.

Although theoretical 'Q-pool' behaviour has been observed in submitochondrial particles oxidizing either succinate or NADH (Kröger & Klingenberg, 1973a) and in reconstituted systems (Ragan & Heron, 1978, and the present paper), it has been reported that deviations from the theory occur during simultaneous oxidation of succinate and NADH by submitochondrial particles (Gutman, 1977). We had hoped that a simultaneous reconstitution of NADH- and L-3-glycerophosphate-cytochrome *c* oxidoreductase activities would enable us to explore the association of two dehydrogenases with Complex III and also to answer some of the questions concerning possible stoichiometric associations. Unfortunately, this did not prove possible, because of the widely different conditions

needed to reconstitute Complex I or L-3-glycerophosphate dehydrogenase with Complex III. In any case, more detailed studies of the reconstitution would need a homogeneous preparation of L-3-glycerophosphate dehydrogenase which had retained its full complement of FAD and other possible redox centres.

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