Solubilization of the alternative oxidase of cuckoo-pint (*Arum maculatum*) mitochondria

Stimulation by high concentrations of ions and effects of specific inhibitors

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1. Selective solubilization of cyanide- and antimycin-insensitive duroquinol oxidase activity from cuckoo-pint (*Arum maculatum*) mitochondria was achieved using taurocholate. Inhibitor-sensitivities and water-forming DQH₂ (tetramethyl-*p*-hydroquinone, reduced form):O₂ stoichiometry were the same for the alternative oxidase of intact *Arum* mitochondria. 2. Cyanide-insensitive oxidation of DQH₂ by intact and solubilized mitochondria was stimulated by up to four-fold by high concentrations of anions high in the Hofmeister series, such as phosphate, sulphate or citrate. Optimal (0.7M) sodium citrate increased $V_{\max}$ for DQH₂ oxidation by the solubilized preparation from 450 to 2400 nmol of O₂·min⁻¹·mg of protein⁻¹ and decreased the apparent K_m for DQH₂ from 0.53 to 0.38 mM. 3. Inhibition of solubilized DQH₂ oxidase activity by CLAM (*m*-chlorobenzhydroxamic acid) and SHAM (salicylhydroxamic acid) was mixed competitive/non-competitive, with apparent inhibition constants for CLAM of 25 μ M (K_i) and 81 μ M (K_i) and for SHAM of 53 μ M (K_i) and 490 μ M (K_i). Propyl gallate and UHDBT were non-competitive inhibitors with respect to DQH₂ (apparent K_i = 0.3 μ M and 12 nM respectively). 4. Low concentrations of C₁₈ fatty acids selectively inhibited cyanide-insensitive oxidation by intact and solubilized mitochondria, and inhibition was reversed by 1% (w/v) bovine serum albumin. Inhibition was competitive with DQH₂, suggesting that fatty acids interfere reversibly with the binding of DQH₂ to the oxidase. 5. These results tend to support the view that quinol oxidation by the alternative pathway of *Arum maculatum* mitochondria is catalysed by a quinol oxidase protein, rather than by a non-enzymic mechanism involving fatty acid peroxidative reactions [Rustin, Dupont & Lance (1983) *Trends Biochem. Sci.* **8**, 155–157; (1983) *Arch. Biochem. Biophys.* **225**, 630–639].

The nature of the non-phosphorylating cyanide- and antimycin A-insensitive oxidase present in many higher-plant mitochondria remains poorly

understood. There is, however, general agreement that the product of dioxygen reduction by the oxidase is water (Huq & Palmer, 1978*a,c*; Rich, 1978). Operation of this oxidase, which branches from the cytochrome pathway at the level of ubiquinone (Huq & Palmer, 1978*b*), seems not to generate specific e.p.r. (Cammack & Palmer, 1977; Rich *et al.*, 1977) or optical signals (Bendall & Bonner, 1971). Consequently the oxidase tends to be identified by the sensitivity of oxygen uptake to inhibitors such as benzhydroxamic acids, for example SHAM and CLAM (Schonbaum *et al.*, 1971), propyl gallate (Siedow & Girvin, 1980), chloroquine (James & Spencer, 1982) or dibromo-

Abbreviations used: CLAM, *m*-chlorobenzhydroxamic acid; DCPIP, 2,6-dichlorophenol-indophenol; DQ, tetramethyl-*p*-benzoquinone (oxidized form); DQH₂, tetramethyl-*p*-hydroquinone (reduced form); FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; SHAM, salicylhydroxamic acid; TMPD, *NNN'*-tetramethyl-*p*-phenylenediamine; UHDBT, 5-(*n*-undecyl)-6-hydroxy-4,7-dioxobenzothiazole.

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thymoquinone (Siedow *et al.*, 1978) and insensitivity to inhibitors of the cytochrome pathway such as cyanide or antimycin A. Fairly recently Cook & Cammack (1983) have reported that UHDBT, a quinone analogue inhibitor of both mitochondrial bc_1 and photosynthetic b_6f complexes (for a review, see Hauska *et al.*, 1983), is also a very potent inhibitor of the alternative pathway of cuckoo-pint (*Arum maculatum*) mitochondria.

Mitochondria from mature *Arum* spadices are capable of very rapid rates of cyanide-insensitive oxidation. Rates of exogenous NADH oxidation of 3000 nmol of $O_2 \cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$ have been reported (Cammack & Palmer, 1977), and duroquinol, ubiquinol-1 and menadiol are also rapidly oxidized. Quinol oxidase activity with similar inhibitor-sensitivities to the alternative pathway has been solubilized from *Arum* mitochondria with deoxycholate (Rich, 1978) and Lubrol (Huq & Palmer, 1978c, 1981). Characterization of the oxidase has been rather slow because of its thermolability (Lance *et al.*, 1978) and the short seasonal availability of *Arum* spadices, but a preparation free of cytochrome and e.p.r.-detectable iron-sulphur clusters, but containing significant amounts of copper and flavoprotein, has been reported (Huq & Palmer, 1978c), and Bonner & Rich (1983) have reported that e.p.r.-silent spin-coupled copper may be involved in operation of the oxidase.

However, a new hypothesis has fairly recently been proposed that questions the participation of an enzyme in the oxidation of ubiquinol by the alternative pathway (Rustin *et al.*, 1983a). In this model, quinol oxidation is catalysed by free radicals of fatty acids, arising from lipid peroxidation, which cycle between oxygenated peroxy and deoxygenated radical forms. In support of this scheme, duroquinol was reported to be co-oxidized with linoleic acid in a cyclic process in the presence of lipoxigenase. This system showed similar inhibitor-sensitivity to the alternative pathway and had a quinol-to-oxygen stoichiometry consistent with water as the end product (Rustin *et al.*, 1983b).

In the present paper we report results obtained when we used a detergent-solubilized preparation of *Arum maculatum* mitochondria to follow the kinetics of duroquinol oxidation and inhibition by SHAM, CLAM, *n*-propyl gallate, UHDBT and non-esterified fatty acids. We also report the effects of high concentrations of ions of the Hofmeister series on the stability and activity of this solubilized cyanide-insensitive DQH_2 oxidase preparation. Results of both of these studies support the view that in *Arum* mitochondria quinol oxidation is catalysed by a highly active quinol oxidase protein.

Materials and methods

Isolation of mitochondria

Mitochondria were isolated, from mature spadices of *Arum maculatum* collected from the wild, by the method of Cammack & Palmer (1977). Isolated mitochondria subsequently treated with detergent were stored in liquid N_2 (77K) until use. Storage in this way did not cause significant loss of KCN-insensitive respiratory activity over a period of 1 month. Protein was determined, after solubilization in 10% (w/v) sodium deoxycholate, by the method of Lowry *et al.* (1951). Bovine serum albumin (fraction V, Sigma) was the standard used.

Solubilization of mitochondria

All operations were carried out at 4°C. Mitochondria were diluted to 16–18 mg of protein $\cdot \text{ml}^{-1}$ with 10 mM- KH_2PO_4 /KOH buffer, pH 7.0, and sodium taurocholate was added to a final detergent/protein ratio of 3:5 (w/w). After gentle mixing for 5 min the solution was centrifuged at 100000g for 45 min at 4°C in a Spinco model L centrifuge. The resulting clear yellow supernatant was retained and kept on ice until use.

Substrates and inhibitors

Fatty acids were emulsified in 20 mM-Hepes buffer, pH 7.0, containing 0.66% (v/v) Tween-80, by sonication for 30 s with a Dawe Soniprobe model 7530A tuned to an output of 3–4 A at setting 3. Samples were immersed in an ice bath during sonication.

Other inhibitors were added from solutions in ethanol or dimethyl sulphoxide such that the final concentration of solvent did not exceed 1% (v/v). In the absence of a published absorption coefficient for UHDBT, solutions of this compound were prepared by weight.

Duroquinol solutions were prepared in acidified dimethyl sulphoxide and not stored. The concentration of the stock solution was determined spectrophotometrically by causing the duroquinol to auto-oxidize by addition of 0.1 M (final concn.) J -KOH. An absorption coefficient of 12500 litre $\cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (oxidized–reduced) at 265 nm was used (Lawford & Garland, 1972).

Measurement of oxidation rates

Oxygen consumption was measured in a total volume of 1 ml at 25°C in a Rank oxygen electrode (Rank Brothers, Bottisham, Cambridge, U.K.). Assay media are as specified in the legends to the Figures, and inhibitors were added before the addition of substrate to ensure maximum inhibition.

The concentration of oxygen in each of the

media used was measured with an oxygen electrode by titration with an airtight solution of 10 mM (approx. concn.) $\text{Na}_2\text{S}_2\text{O}_4$ in 1 M-Tris, pH 9.0. The exact concentration of $\text{Na}_2\text{S}_2\text{O}_4$ was determined by an identical titration of 10 mM-potassium phosphate buffer, pH 7.0, an oxygen concentration of 240 μM being assumed.

The stoichiometry of duroquinol oxidation to oxygen uptake was measured by comparison of oxygen uptake due to operation of the oxidase, in the presence of 1 mM-KCN, with that due to auto-oxidation of duroquinol at pH 9.5 in the presence of catalase (0.5 mg $\cdot$ ml $^{-1}$; Sigma).

Succinate dehydrogenase activity was measured as phenazine methosulphate-mediated DCPIP reduction by the method of Baginsky & Hatefi (1969), with the addition of 0.04% (v/v, final concn.) Triton X-100. Samples were activated by preincubation with 0.15 mM-ATP for 5 min to relieve inhibition of succinate dehydrogenase.

Chemicals

UHDBT and CLAM were kindly given by Dr. N. Packham, Imperial College of Science and Technology, London, U.K., and Dr. I. Ericson, University of Umeå, Umeå, Sweden, respectively. DCPIP (sodium salt), n-propyl gallate, linoleic acid (sodium salt), linolenic acid (non-esterified acid), oleic acid (sodium salt) and taurocholic acid (sodium salt) were from Sigma. DQH₂ was from K and K Laboratories, Plainview, NY, U.S.A. SHAM was from Ralph N. Emanuel, Wembley, Middx., U.K.

Other chemicals were from BDH Chemicals, Poole, Dorset, U.K., Hopkin and Williams, Chadwell Heath, Essex, U.K., or Sigma Chemical Co., Poole, Dorset, U.K., and were of the highest grade available.

Results

Properties of the solubilized preparation

Treatment of *Arum maculatum* mitochondria with taurocholate yielded a clear yellow fraction (9–13 mg of protein $\cdot$ ml $^{-1}$) containing increased specific DQH₂ oxidase activity over that found in intact mitochondria (Table 1). This activity was stable for several hours if the preparation was kept on ice. Addition of 0.5 mM-DQ to mediate electron transfer between dehydrogenase and oxidase indicated the presence of NADH dehydrogenase and some succinate dehydrogenase in the solubilized fraction. The presence of succinate dehydrogenase was confirmed by DCPIP reduction, because the assay conditions used to optimize KCN-insensitive DQH₂ oxidation (Fig. 1 and Table 2 below) caused an inhibition of succinate oxidase activity. Oxidation of NADH or succinate in the absence of

Table 1. *Properties of intact and solubilized Arum mitochondria*

(a) Oxygen uptake was measured in a medium consisting of 0.7 M-sodium citrate and 0.2 μM -FCCP, pH 7.0. Rates are nmol of $\text{O}_2 \cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$ or per 4 reducing equivalents. (b) Cytochrome was measured by using the wavelength pairs of Lance & Bonner (1968).

(a) Oxygen uptake

Substrate	Oxygen uptake rate	
	Mitochondria	Solubilized preparation
1 mM-DQH ₂ + 1 mM-KCN	1762	2390
0.75 mM-NADH + 1 mM-KCN	691	69
0.75 mM-NADH + 0.5 mM-DQ + 1 mM-KCN	693	642
10 mM-Succinate + 1 mM-KCN	62 (see the text)	0
10 mM-Succinate + 0.5 mM-DQ + 1 mM-KCN	61	10
Succinate dehydrogenase	510	195
5 mM-Ascorbate + 0.5 mM-TMPD	75	2

(b) Cytochrome content

Cytochrome	Content (nmol $\cdot$ mg of protein $^{-1}$)	
	Mitochondria	Solubilized preparation
<i>b</i>	0.301	0.105
<i>c</i>	0.194	0.045

DQ was slow, presumably because detergent treatment caused separation of the oxidase from dehydrogenase complexes, so preventing ubiquinol-mediated electron transfer. The preparation contained very little cytochrome oxidase activity and less of the *b*- and *c*-type cytochromes than in intact mitochondria (Table 1). The properties of the taurocholate-solubilized preparation are similar to those reported by Rich (1978) after treatment with deoxycholate at a detergent/protein ratio of 4:1 (w/w).

The stoichiometry of oxygen uptake was unaffected by addition of 1 mM-cyanide (to inhibit residual catalase activity) and was consistent with water being the end product, in agreement with the reports of Huq & Palmer (1978c) and Rich (1978).

We have previously experienced difficulty in obtaining reliable kinetic measurements of DQH₂ oxidation by intact *Arum* mitochondria. This may be due to problems of partition of DQH₂ between

aqueous and membrane phases. We therefore used the soluble preparation for kinetic measurements, since oxidation of DQH_2 by solubilized and intact mitochondria showed similar inhibitor-sensitivities, stoichiometry and specific activity.

Effects of high concentrations of ions on DQH_2 oxidation

High concentrations of sodium citrate, up to an optimum of 0.7 M, were found to enhance cyanide-insensitive DQH_2 oxidation by both intact and solubilized mitochondria (Fig. 1) while retaining complete sensitivity to $1\mu\text{M}$ -UHDBT and $\text{DQH}_2:\text{O}_2$ stoichiometry consistent with water production. As DQH_2 oxidation by both intact and solubilized mitochondria was stimulated, it is unlikely that the effect is due to changes in the solubilizing properties of taurocholate (critical micelle concentration and micelle size) known to occur with increasing ionic strength (Carey & Small, 1969). This view was supported by the observation that inclusion of 1% (w/v) sodium taurocholate in the assay of the solubilized preparation did not affect the oxidation rate.

The effect of sodium citrate on the kinetics of DQH_2 oxidation (Fig. 2) was simultaneously to increase V_{max} (from 450 to 2400 nmol of $\text{O}_2 \cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$) and decrease K_m (from 0.53 to 0.38 mM).

Fig. 1. Effect of high concentration of sodium citrate on DQH_2 oxidation by intact and solubilized mitochondria. Initial rates of oxidation on addition of 1 mM- DQH_2 were measured at 25°C in buffers of increasing sodium citrate concentration at pH 7.0. For zero citrate concentration, 10 mM-sodium phosphate, pH 7.0, was the buffer used. O, Solubilized preparation (0.5 mg of protein · ml $^{-1}$); ●, non-solubilized mitochondria (0.34 mg of protein · ml $^{-1}$) + 1 mM-KCN.

With intact mitochondria, only KCN-insensitive oxidation of DQH_2 was stimulated by the presence of high citrate concentrations (Table 2). Oxidation of succinate, NADH and DQH_2 + SHAM were inhibited to different extents by sodium citrate. Inhibition of NADH dehydrogenase may have been by chelation of bivalent cations by citrate, particularly Ca^{2+} (Palmer & Coleman,

Fig. 2. Effect of high sodium citrate concentrations on the kinetics of cyanide-insensitive DQH_2 oxidation.

Assay conditions were as shown in Fig. 3, but with sodium citrate concentrations as indicated: ▲, 0.1 M; △, 0.2 M; ●, 0.4 M; ○, 0.7 M.

Table 2. Substrate oxidation by Arum mitochondria in the presence of 0.7 M-sodium citrate

Linear rates of oxygen uptake were measured in (i) standard medium (0.3 M-sucrose/10 mM-Tris/KOH/5 mM- KH_2PO_4 /5 mM- MgCl_2 /0.2 μM -FCCP, pH 7.2), or (ii) 0.7 M-sodium citrate/0.2 μM -FCCP, pH 7.0. Inhibitor concentrations were 1 mM.

Substrate	Medium	Rate (nmol of $\text{O}_2 \cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$)	
		Standard	Citrate
NADH (0.75 mM)		872	792
	+ KCN	872	824
	+ SHAM	214	103
Succinate (10 mM)		722	179
	+ KCN	668	179
	+ SHAM	450	97
DQH_2 (1 mM)		765	1763
	+ KCN	388	1704
	+ SHAM	186	124

1974), but the cause of the large inhibition of succinate dehydrogenase is not known. Thus, in intact mitochondria, limitation at the dehydrogenase level may obscure increases in the activity of the oxidase caused by citrate. Oxidation of citrate itself was not detected under these conditions.

Oxidation rates obtained with the solubilized preparation in the presence of high concentrations of other ions are shown in Table 3. Of the anions tested, citrate, phosphate and sulphate were particularly stimulatory: the rate when assayed in 1 M-sodium citrate was four times higher than that found in 10 mM-potassium phosphate. Tartrate also increased the oxidation rate, but acetate and chloride had little effect. Cations were less effective at increasing the rate, K^+ and Na^+ stimulating more than Li^+ or Mg^{2+} . The specificity of this effect for different ionic species indicates that these observations are not simply due to high ionic strength or osmolarity.

High concentrations of both cations and anions have been known for many years to have marked effects on conformational stability and solubility of biopolymers [see reviews by Franks & Eagland (1975) and Von-Hippel & Schliech (1969)]. The effectiveness of different ions in promoting these transitions is often similar to the empirical Hofmeister series, originally derived from studies on the effectiveness of cations and anions in the precipitation of serum globulin proteins. In order of increasing effectiveness as precipitants, the ions

used in Table 3 are ranked in the Hofmeister series:

Cations: $Li^+ > Na^+ > K^+ > Mg^{2+}$

Anions: sulphate > phosphate > acetate
> citrate > tartrate > chloride

It can be seen that the order of ions in this series is similar to, but not identical with, the degree to which cyanide-insensitive DQH_2 oxidation was stimulated (Table 2). Specific differences were that Li^+ and acetate had less effect than would be predicted from the Hofmeister series.

High ionic concentrations also affect the solubility of many small molecules (Long & McDevitt, 1952). However, since the effect of ions high in the Hofmeister series is to decrease the solubility of hydroquinone and ubiquinone (Linderstrom-Lang, 1923) and the maximum concentration of DQH_2 used in the present study was 1 mM, it is unlikely that changes in solubility of DQH_2 are relevant to the effects described here.

An increase in rate in the presence of high concentrations of anions has also been reported for O_2 evolution by isolated spinach (*Spinacia oleracea*) chloroplasts (Stewart, 1982), but, in contrast with that report, sodium citrate (0.7 M) was not found to protect DQH_2 oxidase activity from denaturation by heat or by concentrations of the chaotropic agent $NaNO_3$ of up to 1 M (results not shown).

Table 3. Effects of ions on KCN-insensitive oxidation of DQH_2

Initial rates of oxidation of 1 mM- DQH_2 by solubilized *Arum* mitochondria (0.3–0.4 mg of protein·ml⁻¹) were measured at pH 7.0. All assay media contained 10 mM-potassium phosphate as buffer.

Addition to 10 mM-potassium phosphate	Concn. (M)	[O ₂] (μM)	Rate (nmol of O ₂ ·min ⁻¹ ·mg of protein ⁻¹)
Control	0	240	550
Sodium sulphate	0.5	154	822
	1.0	119	1856
Potassium phosphate	0.5	167	901
	1.0	130	1811
Sodium acetate	0.5	183	432
	1.0	147	425
Sodium citrate	0.5	142	2148
	1.0	95	1973
Sodium potassium tartrate	0.5	162	770
	1.0	103	1046
LiCl	0.5	194	665
	1.0	158	632
NaCl	0.5	201	725
	1.0	159	848
	2.0	107	899
KCl	0.5	199	696
	1.0	168	723
	2.0	118	865
MgCl ₂	0.5	214	537
	1.0	166	464

Effects of inhibitors

(1) *Inhibition by SHAM and CLAM.* Benzhydroxamic acids were introduced as inhibitors of the alternative pathway by Schonbaum *et al.* (1971). The inhibition by both CLAM and SHAM of DQH_2 oxidation by the solubilized preparation was found to be mixed competitive/non-competitive in type (Fig. 3). Secondary plots gave values for apparent inhibition constants for CLAM of $25\text{ }\mu\text{M}$ (K_i) and $81\text{ }\mu\text{M}$ (K_i), and for SHAM of $53\text{ }\mu\text{M}$ (K_i) and $490\text{ }\mu\text{M}$ (K_i). It was of interest that both DQH_2 oxidation and its inhibition by all compounds studied showed saturation kinetics consistent with binding of substrates and inhibitors to specific sites. These results suggest that both SHAM and CLAM inhibit by binding to either the free enzyme (K_i) or the enzyme- DQH_2 complex (K_i), though with both inhibitors binding to the enzyme-quinol complex was less tight than to the enzyme alone.

(2) *Inhibition by n-propyl gallate and UHDBT.* In contrast with SHAM and CLAM, propyl gallate was found to inhibit DQH_2 oxidation non-competitively with respect to DQH_2 , with an apparent K_i of $0.3\text{ }\mu\text{M}$ (Fig. 4a). As with SHAM and CLAM, this result suggests that propyl gallate is able to bind at a separate site from DQH_2 , but unlike SHAM and CLAM the presence of bound DQH_2 does not affect the binding of propyl gallate.

This strengthens the view that separate inhibitor- and substrate-binding sites exist on the oxidase.

The quinone analogue inhibitor UHDBT, reported by Cook & Cammack (1983) to inhibit succinate and NADH oxidation by *Arum* mitochondria at very low concentrations, was found to inhibit DQH_2 oxidation by the solubilized preparation non-competitively (apparent $K_i = 12\text{ nM}$; Fig. 4b). This result is rather surprising, since a quinone analogue inhibitor might have been expected to inhibit competitively with respect to a quinol substrate.

(3) *Inhibition by non-esterified fatty acids.* A near doubling of NADH and succinate oxidation by mung bean (*Phaseolus aureus*) mitochondria has been reported to occur in the presence of bovine serum albumin (Rich & Bonner, 1978). Similarly, non-esterified fatty acids were found to inhibit the operation of the alternative pathway (Rustin *et al.*, 1984). In view of the recent suggestion that quinol oxidation via the alternative pathway is catalysed by lipid-peroxidative processes (Rustin *et al.*, 1983a), the effects of non-esterified fatty acids on solubilized and intact mitochondria were compared. The effect of linoleic acid on DQH_2 oxidation by intact mitochondria via both the cytochrome and alternative pathways is shown in Fig. 5. Young *Arum* spadices of the α - β stage (James & Beevers, 1950) were used in this experiment, since these mitochondria had cytochrome and alternative pathways of similar activity. At

Fig. 3. Inhibition of cyanide-insensitive DQH_2 oxidation by (a) CLAM and (b) SHAM

Initial rates of oxidation by a taurocholate-solubilized mitochondrial preparation (0.30 – 0.4 mg of protein $\cdot\text{ml}^{-1}$) were measured in a medium consisting of 0.7 M -sodium citrate, pH 7.0 , at 25°C , at a range of DQH_2 and inhibitor concentrations. (a) $\circ$, No inhibitor; CLAM at: $\bullet$, $20\text{ }\mu\text{M}$; $\triangle$, $50\text{ }\mu\text{M}$; $\blacktriangle$, $100\text{ }\mu\text{M}$; $\square$, $200\text{ }\mu\text{M}$. (b) $\circ$, No inhibitor; SHAM at: $\bullet$, $100\text{ }\mu\text{M}$; $\triangle$, $200\text{ }\mu\text{M}$; $\blacktriangle$, $400\text{ }\mu\text{M}$.

Fig. 4. Inhibition of cyanide-insensitive DQH_2 oxidation by (a) *n*-propyl gallate and (b) UHDBT. Assay conditions were as shown in Fig. 3. (a) ○, No inhibitor; *n*-propyl gallate at: ●, 0.1 μM; △, 0.2 μM; ▲, 0.5 μM; □, 1 μM. (b) ○, No inhibitor; UHDBT at: ●, 68 nM; △, 101 nM; ▲, 135 nM.

Fig. 5. Inhibition by linoleic acid of DQH_2 oxidation by α - β stage *Arum* mitochondria

Initial rates of oxidation of 1 mM-DQH by *Arum* mitochondria of the α - β stage (James & Beevers, 1950) were measured in the standard assay medium of Table 2. ●, +1 mM-KCN; ○, +1 mM-SHAM; △, +1 mM-KCN + 1% (w/v) bovine serum albumin.

Fig. 6. Inhibition of cyanide-insensitive DQH_2 oxidation by C_{18} fatty acids

Initial rates of oxidation on addition of 1 mM-DQH₂, by a solubilized preparation (0.43 mg of protein · ml⁻¹), was measured at 25°C in 0.7 M-sodium citrate, pH 7.0, in the presence of increasing fatty acid concentrations. ○, Linolenic acid (C_{18:3}); ●, linoleic acid (C_{18:2}); △, oleic acid (C_{18:1}).

these concentrations (up to 50 μM, or 0.125 μmol of fatty acid · mg of protein⁻¹), linoleic acid selectively inhibited cyanide-insensitive DQH_2 oxidation without causing significant inhibition of KCN-sensitive DQH_2 oxidation. Inhibition was largely reversed by the presence of 1% (w/v) bovine serum albumin. Oxidation of succinate or NADH by intact mitochondria via the alternative, but not the cytochrome, pathway was similarly inhibited by linoleic acid (results not shown). Since the

alternative and cytochrome pathways branch at the level of ubiquinone, these results show selective inhibition by linoleic acid of quinol oxidation via the alternative pathway. It was found that

Fig. 7. Inhibition of cyanide-insensitive DQH_2 oxidation by linoleic acid

Assay conditions were as shown in Fig. 3. ○, No inhibitor; linoleic acid at: ●, 10 μM ; △, 50 μM ; ▲, 100 μM ; □, 200 μM .

oleic ($C_{18:1}$), linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$) acids were equally effective inhibitors of DQH_2 oxidation by the solubilized preparation (Fig. 6) and that inhibition by linoleic acid was essentially competitive with respect to DQH_2 (Fig. 7). The lack of inhibition of DQH_2 oxidation via the cytochrome pathway and the small amount of fatty acid required for inhibition (in comparison with the concentrations of DQH_2 added as substrate) make it unlikely that fatty acids inhibit by sequestering DQH_2 away from the oxidase into mixed fatty acid- DQH_2 micelles.

Since intact and solubilized mitochondria were similarly inhibited, it seems that non-esterified fatty acid can interfere reversibly with the interaction of quinol with the oxidase.

Discussion

The present results show that the selective solubilization of *Arum maculatum* mitochondria by taurocholate produces a suitable soluble system for study of the kinetics of the alternative oxidase in the absence of cytochrome oxidase-mediated quinol oxidation. Our interest in inhibitors of the alternative oxidase was firstly to attempt to distinguish between quinol oxidation catalysed by a protein oxidase and that catalysed by a cycling free-radical system (Rustin *et al.*, 1983*a,b*). It was thought that the free-radical system would be unlikely to give the simple inhibition patterns expected for an enzymic system, because of the lack of inhibitor- or substrate-binding sites. Our results showed simple enzymic-inhibition patterns for all inhibitors tested and therefore tend to

support the enzymic hypothesis of the alternative oxidase.

Benzhydroxamic acids inhibit several other enzymes, such as mushroom tyrosinase (Rich *et al.*, 1978), peroxidase (Schonbaum, 1973) and lipoxygenase (Siedow & Girvin, 1979). Inhibition of tyrosinase was reported to be competitive with reducing substrate, but not with O_2 , and possible competition between hydroxamate and ubiquinol for binding sites was suggested (Rich *et al.*, 1978) to explain the dependence of apparent K_i for inhibition of cyanide-insensitive respiration by SHAM on electron flux (Tomlinson & Moreland, 1975). Cottingham & Moore (1983) have reported competitive inhibition by SHAM of DQH_2 oxidation by intact *Arum* mitochondria. However, our results with both SHAM and CLAM, obtained with a solubilized system, are more consistent with mixed competitive/non-competitive than simple competitive inhibition. Mixed inhibition may provide an alternative explanation for the non-linear Dixon plot reported by Cottingham & Moore (1983) for SHAM inhibition of NADH oxidation by *Arum* mitochondria. From this plot, two limiting apparent K_i values were derived of 8 μM and 275 μM at low and high SHAM concentrations respectively. Values of 53 μM (apparent K_i) and 490 μM (apparent K_i) were obtained in the present study, suggesting that, at low SHAM concentration when the ubiquinone pool is relatively oxidized, SHAM will bind predominantly to the free enzyme (K_i), with high affinity. At high SHAM concentration the ubiquinone pool will become reduced, and SHAM will bind to the enzyme-substrate form (K_i), with low affinity. The lower affinities for SHAM reported here compared with those of Cottingham & Moore (1983) may be due to partition of hydroxamate into the membrane phase of intact mitochondria.

The mechanism of hydroxamate binding to, and inhibition of, the alternative oxidase is not clear, but metal-ion chelation (Anderegg *et al.*, 1963) has been discounted (Rich *et al.*, 1977). Other suggestions have been polyfunctional hydrogen-bonding (Schonbaum, 1973) or formation of a 'charge-transfer complex' between hydroxamate and an acceptor group on the enzyme (Rich *et al.*, 1978). The latter theory derives from the ability of hydroxamates to undergo one-electron oxidation to form short-lived radicals (Ramsbottom & Waters, 1966).

Grover & Laties (1978) reported that CLAM and SHAM binding are mutually exclusive in sweet-potato (*Ipomoea batatas*) mitochondria, suggesting that both compounds compete for identical binding sites. The finding of mixed inhibition for both SHAM and CLAM is consistent with an identical binding site, but not with the

suggestion that these compounds act as general competitors with quinol (Rich *et al.*, 1978), since benzhydroxamate and DQH₂ binding is not mutually exclusive and so probably occur at different sites.

The finding that UHDBT, a quinone analogue, was non-competitive inhibitor with respect to DQH₂ was surprising. However, it may be significant that both UHDBT and dibromothymoquinone are redox-active inhibitors of the alternative pathway, capable of forming relatively stable semiquinones [$E'_m(Q^{\cdot-}/Q) = -70$ and $+70$ mV respectively] (Rich & Bendall, 1980). Inhibition may therefore, as suggested for benzhydroxamates, be due to the ability of UHDBT and dibromothymoquinone to form a charge-transfer complex with an as-yet-unidentified centre of the enzyme, rather than acting simply as a competitive quinone-analogue inhibitor. Whatever the mode of inhibition, it is apparent that sites of hydroxamate, propyl gallate and UHDBT inhibition are different from the site of DQH₂ binding.

Inhibition by linoleic acid of the alternative pathway of substrate oxidation and reversal of inhibition by bovine serum albumin have been reported for intact *Arum* mitochondria (Rustin *et al.*, 1984). The reversal of inhibition is due to the well-known binding of fatty acids to bovine serum albumin. Rustin *et al.* (1984) suggested that, according to the non-enzymic hypothesis of the alternative oxidase (Rustin *et al.*, 1983a), fatty acids may inhibit the alternative pathway by causing fluidity changes in the mitochondrial inner membrane. These changes would disrupt the oxidative interaction of the lipid free radicals with ubiquinol. The inhibition of the oxidase in the solubilized form and the finding of competitive inhibition with respect to DQH₂ reported here suggest the alternative explanation, namely that fatty acids bind reversibly to the quinol-binding site of the oxidase.

High concentrations of anions such as phosphate, sulphate and citrate were found to enhance DQH₂ oxidation by up to 400% compared with the rate when assayed in 10 mM-potassium phosphate. The increased rate was mediated by the alternative oxidase, since correct inhibitor-sensitivities and DQH₂:O₂ stoichiometries were preserved. Although it is unlikely to be of physiological significance, this stimulation of rate was of interest (a) in order to optimize assay conditions for the further purification of the oxidase, and (b) since the stimulation provides further evidence for the existence of a protein oxidase, by analogy with other proteins similarly affected. Unfortunately one of the problems with isolation of the oxidase is its lability (Lance *et al.*, 1978), and 0.7 M-sodium citrate did not significantly protect against de-

naturation by heat or by high concentrations of the chaotrope NaNO₃.

The order of effectiveness of ions in stimulating the oxidase paralleled closely the Hofmeister series, with some differences (noted above). The effects of electrolytes high in the Hofmeister series on soluble proteins is often to stabilize native conformation, as with ribonuclease (Jencks, 1969). Applications of these effects to membrane proteins include the stabilization of mammalian mitochondrial complex II by phosphate, sulphate, citrate and acetate (Jencks, 1969; Davis & Hatefi, 1972) and the stimulation and stabilization of O₂ evolution by spinach chloroplasts in the presence of ions high in the Hofmeister series (Stewart, 1982). At the low end of the series, washing with 2 M-NaBr has been reported to detach coupling factor from thylakoid membranes (Nelson, 1980).

The molecular basis for the effects of these electrolytes is not fully understood. However, most explanations involve modification of the organization of solvent around potentially exposed groups on the protein, increasing or decreasing the free energy of transfer of these groups to the exterior, through effects on local water structure (Von Hippel & Schliech, 1969). Franks (1977) has described three solvent regions around a solvated protein: 'A'-shell water, or the primary hydration sphere of the protein; 'C'-phase or bulk-phase water, and an intermediate 'B'-shell in which water structure reflects perturbations resulting from the proximity of the amphipathic protein molecule. It was suggested that effects of electrolytes on protein conformational stability are mediated through effects on the structure of the 'B'-shell water. The increase in V_{max} and decrease in K_m of the oxidase suggests that increasing citrate concentration stabilizes a protein conformation of increased catalytic efficiency and alters the microenvironment of the DQH₂-binding site such that interaction of DQH₂ with the oxidase is promoted. Further purification of the oxidase will be needed to determine whether the conformational stability being altered is that of a single protein or of a protein complex, but it seems that factors affecting thermal stability of the oxidase, perhaps loss of bound copper (Bonner & Rich, 1983), are not modified by high ion concentrations.

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