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The Oxidation of Ubiquinol by the Isolated Rieske Iron–Sulfur Protein in Solution[1]

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The pre-steady-state redox reactions of the Rieske iron–sulfur protein isolated from beef heart mitochondria have been characterized. The rates of oxidation by *c*-type cytochromes is much faster than the rate of reduction by ubiquinols. This enables the monitoring of the oxidation of ubiquinols by the Rieske protein through the steady-state electron transfer to cytochrome *c* in solution. The pH and ionic strength dependence of this reaction indicate that the ubiquinol anion is the direct reductant of the oxidized cluster of the iron–sulfur protein. The second electron from ubiquinol is diverted to oxygen by the isolated Rieske protein, and forms oxygen radicals that contribute to the steady-state reduction of cytochrome c. Under anaerobic conditions, however, the reduction of cytochrome *c* catalyzed by the protein becomes mechanicistically identical to the chemical reduction by ubiquinols. The present kinetic work outlines that: (i) the electron transfer between the ubiquinol anion and the Rieske cluster has a comparable rate when the protein is isolated or inserted into the parent cytochrome *c* reductase enzyme; (ii) the Rieske protein may be a relevant generator of oxygen radicals during mitochondrial respiration.

The reduced form of the quinones involved in energy-transducing redox chains are oxidized by several oxidoreductase enzymes inserted into biological membranes (1). Two types of such enzymes catalyze the electron transfer from quinols to water-soluble one-electron acceptors: ubiquinol–cytochrome *c* reductase (bc_1 complex) in mitochondria and bacteria, and plastoquinol–plastocyanin reductase (b_6f complex) in chloroplasts (1, 2). Both of these reductases possess a high-potential 2Fe2S subunit that is usually called Rieske protein (3) after its discoverer (4, 5). The Rieske protein is believed to directly oxidize ubiquinol or plastoquinol and then transfer electrons to the *c*-type cytochrome (either cytochrome c_1 or cytochrome *f*) which is the direct electron donor to the water-soluble acceptors (2, 3, 6).

Several works of pre-steady-state kinetics have elucidated the functional role of the Rieske iron–sulfur protein within the parent reductase (3, 6, 7), a role that is crucial in the cyclic models that are now generally accepted for the redox mechanism of the bc_1 complex (see (8, 9) for recent reviews). Extensive studies have been undertaken on the oxidation of quinols by *c*-type cytochromes in solution, reaction considered as a model for the physiological mechanism of ubiquinol oxidation within the reductases (6, 10–13). The basic conclusion derived from these studies is that the anionic form of ubiquinol is the effective reductant, whereas the semiquinone form(s) do not directly react with the electron acceptor (6, 11, 12). It is usually assumed that the same concepts apply to the oxidation of quinols catalyzed by the Rieske protein (3, 6, 9, 10, 12, 13). However, apart from few preliminary data (4, 14), the redox reactions of the isolated Rieske protein have not been characterized yet. Here we report a complete evaluation of the electron-transfer reactions of the beef heart Rieske protein that contribute to a better understanding of the mechanism of ubiquinol oxidation.

MATERIALS AND METHODS

Preparations and materials. The bc_1 complex was purified from beef heart mitochondria as previously described (15). The Rieske iron–sulfur protein was isolated by the procedure of Shimomura *et al.* (16) and was concentrated by ultradialysis after elution from the phenyl-Sepharose column. This preparation was contaminated only by small amounts of the core protein subunits and was capable of reconstituting 50 to 80% of the reductase activity in the iron–sulfur-depleted enzyme (17). For the pre-steady-state measurements, the concentrated protein was oxidized with solid potassium ferricyanide and subsequently desalted in a Sigma PD10 column equilibrated with

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the same phosphate buffer used in the assays. The iron–sulfur protein-depleted complex and isolated cytochrome c_1 were obtained as described in Refs. (16, 17) and (14), respectively.

Cytochrome *f* (turnip or spinach), cytochrome *c* (horse heart, type VI), superoxide dismutases (bovine and *Escherichia coli*), glucose oxidase, and catalase were purchased from Sigma. Ubiquinone-2 was donated by Eisai Co. (Tokyo, Japan) and reduced by the method of Rieske (18). All the other reagents were of analytical grade.

Determinations. The concentrations of the cytochromes were determined from their optical difference spectrum, reduced minus oxidized, using the following extinction coefficients (for a 1-cm path length): 17.5 mM^{-1} at 542–541 nm for cytochrome c_1 (19); 19 mM^{-1} at 550–540 nm or 13.2 mM^{-1} at 549.5–515 nm for cytochrome *c*; 19 mM^{-1} at 554–542 nm (20) or 105 mM^{-1} at 421–432 nm for cytochrome *f*. Ubiquinols were determined in ethanol at 290 nm with an extinction coefficient of 4 mM^{-1} cm^{-1} (21). The concentration of the iron–sulfur cluster in the isolated Rieske protein was determined from the difference, oxidized minus reduced, of either the optical spectrum at 570–700 nm with an extinction coefficient of 1.5 mM^{-1} cm^{-1} (3–5) or the circular dichroic spectrum at 500–462 nm with an extinction coefficient of 12.4 M^{-1} cm^{-1} (17). These determinations were in consistent agreement with independent measurements of the non-heme iron performed as described by Rieske (5).

Redox assays. The ubiquinol–cytochrome *c* reductase activity was assayed at 25°C and with quasi saturating concentrations of the substrates (typically, 20 μM ubiquinol-2 and 10 μM cytochrome *c*), as described previously (15). The reaction was started by ubiquinol and the absorbance changes were measured in a Sigma Biochem dual-wavelength spectrophotometer equipped with a rapid-mixing apparatus which is able to complete mixing in 0.1 s. The usual buffer was 25 mM potassium phosphate, pH 7.2 (unless otherwise stated), containing 1 mM EDTA and, for removing oxygen from the medium, 10–30 mM glucose plus various concentrations of both glucose oxidase and catalase. When the assays were performed under anaerobic conditions, the mixing apparatus was used only for 2 s upon addition of substance(s) in the medium. Less than 5 μM O_2 was detected under such conditions by an oxygen electrode.

The rates of cytochrome *c* reduction by ubiquinols, both chemical rates and those catalyzed by 0.05–0.2 μM Rieske protein, were usually measured as the tangents to the linear portion of the traces (10–12, 22) (see also Fig. 2A). The alternative measure of the rates which was obtained from the semilogarithmic plot of the time courses yielded values of the second-order rate constants that were in very close agreement with those obtained from the above tangents (V_i). The rates catalyzed by the Rieske protein were always corrected for the chemical rates. The pre-steady-state oxidation of the Rieske protein prereduced with nearly stoichiometric amounts of either sodium ascorbate or ubiquinol-2 was measured at 570–700 nm when ferricyanide was the oxidant or by following the reduction of cytochrome *c* at 549.5–515 nm—where minimal optical interference from the iron–sulfur cluster was detected (22)—when cytochrome *c* was the oxidant. The reduction of the oxidized Rieske protein was measured at 570–700 nm (22).

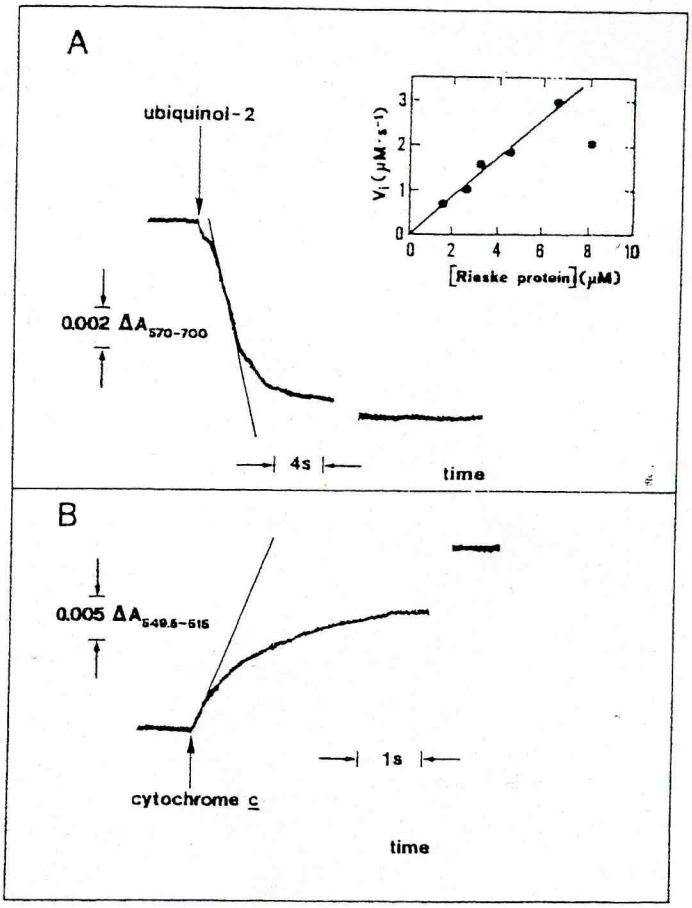

FIG. 1. Pre-steady-state reduction and oxidation of the isolated Rieske protein. (A) The trace shows the time course of the reduction of 5.8 μM oxidized Rieske cluster by 18 μM ubiquinol-2. The broken line at the bottom of the trace corresponds to the final level of reduction. In the insert, the initial rates (V_i) of reduction by 18 μM ubiquinol-2 are plotted as a function of several concentrations of oxidized cluster. (B) Oxidation of the Rieske protein as monitored by the reduction of cytochrome *c* (1.2 μM) by an excess of ascorbate-reduced cluster (5.5. μM). The broken line on the top of the trace indicates the final level of the reaction, which was markedly biphasic (only about 50% of the trace followed the first rapid phase). Note that the time scale is fivefold larger than in (A).

RESULTS

Redox Reactions of the Isolated Rieske Iron–Sulfur Protein

The redox reactions of the isolated Rieske protein can be easily detected from the absorbance changes in the reducible iron of the cluster at 570–700 nm (3) by using a sensitive and rapid spectrophotometric device (Fig. 1 and (22)). The pre-steady-state reactions measured with an excess of reductants (Fig. 1A) or oxidants (e.g., ferricyanide (22)) follow a pseudo-first-order dependence for over 90% of their time course. The rapid oxidation of the prereduced protein by cytochrome *c* (Fig. 1B), however, follows a biphasic time course, mostly because of an overstoichiometric reduction of the Rieske cluster and of the low ratio of the reactants. Nevertheless, also in this case the initial rates are linearly dependent upon the concentration of the reagents (Fig. 1A, insert, and (22)), thus enabling the determination of the rate constant. The various second-order rate constants obtained with different oxidants and reductants are listed in Table I.

Isolated cytochrome c_1, the physiological oxidant of the Rieske 2Fe2S cluster in the bc_1 complex (3, 6, 7), appears to be a poor electron acceptor for the reduced pro-

TABLE I

Second-Order Rate Constants of Several Oxido-Reduction Reactions Involving c-Type Cytochromes, Ubiquinol-2 and the Rieske Protein

Reductant	Oxidant	Rate constant (M^{-1} s^{-1})
Rieske protein red	Ubiquinone-2	n.d.[a]
Rieske protein red	Potassium ferricyanide	1.3×10^4
Rieske protein red	Cytochrome c_1	$1–3 \times 10^{3b}$
Rieske protein red	Cytochrome *c*	1.3×10^5
Rieske protein red	Cytochrome *f*	1.5×10^{6c}
bc_1 complex red	Cytochrome *c*	4.0×10^{8d}
Ubiquinol-2	Cytochrome c_1	n.d.[a]
Ubiquinol-2	Potassium ferricyanide	4.0×10^1
Ubiquinol-2	Cytochrome *c*	1.0×10^2
Ubiquinol-2	Cytochrome *f*	4.0×10^3
Ubiquinol-2	Rieske protein ox	2.2×10^4
Ubiquinol-2	bc_1 complex ox	4.2×10^{8d}
Ascorbate	Rieske protein ox	1.2×10^3

Note. The reactions are measured at pH 7.2 and room temperature.

[a] Rate not detectable after several minutes (see also (4, 14)).

[b] The highest values are seen with excess of ubiquinol-2 that reduces the Rieske protein (14).

[c] Measured by the steady-state assay of ubiquinol-2–cytochrome *f* reductase catalyzed by the Rieske protein in the presence of superoxide dismutase and catalase and with high ubiquinol concentrations (22).

[d] From the steady-state reductase activity of the isolated bc_1 complex, assuming a ping-pong two-site mechanism (15, 23).

tein (Table I). Presumably, the formation of a stoichiometric complex between isolated cytochrome c_1 and isolated Rieske protein (14, 17) prevents the rapid collisional second-order reaction that takes place with the other cytochromes. Moreover, a perturbation of the heme center in cytochrome c_1 is likely to occur upon its isolation, as indicated by its altered circular dichroic spectra (17), and this may affect electron transfer with the Rieske cluster.

Properties of the Ubiquinol-2–Cytochrome c Reductase Catalyzed by the Isolated Rieske Protein

The comparison of the rates of ubiquinol oxidation (Table I) clearly outlines that the Rieske protein is the most efficient oxidant of ubiquinols. Hence, the isolated protein can be utilized as the ideal biological model for studying the mechanism of ubiquinol oxidation. To this end, it is convenient to exploit the catalytic action of the isolated protein on the reduction of cytochrome *c* by ubiquinols (14) (Fig. 2A). Cytochrome *c* is preferred to cytochrome *f* as the electron acceptor in these steady-state measurements because the latter presents a faster reactivity with quinols (Table I) and is difficult to be maintained fully oxidized due to its high midpoint potential (2).

Ubiquinol-2 is the best quinol for characterizing the cytochrome *c* reduction catalyzed by the isolated Rieske protein, as it is the most efficient substrate of the bc_1 complex (15). At difference with the enzymatic reaction (Table I, see also (23)), ubiquinol-2 reduces the isolated Rieske protein with a rate that is one order of magnitude slower than the rate at which the same protein is reoxidized by cytochrome *c* (Table I). Indeed, under the conditions that are usually employed in the enzymatic assay, the steady-state reaction catalyzed by the Rieske protein is basically rate-limited by the oxidation of ubiquinol-2, as indicated by the apparently hyperbolic dependence of the rates on the concentration of cytochrome *c* (Fig. 2B) and the linear dependence of the rates on the ubiquinol concentration (Fig. 2C). When the concentration of cytochrome *c* is kept much lower than that of ubiquinol, a hyperbolic dependence on the ubiquinol concentration is also seen (data not shown), because under such conditions the rate-limiting step has been shifted to the reoxidation of the reduced Rieske cluster by the cytochrome (see (12, 13) for a detailed explanation of this kind of kinetic behavior). The rates are also

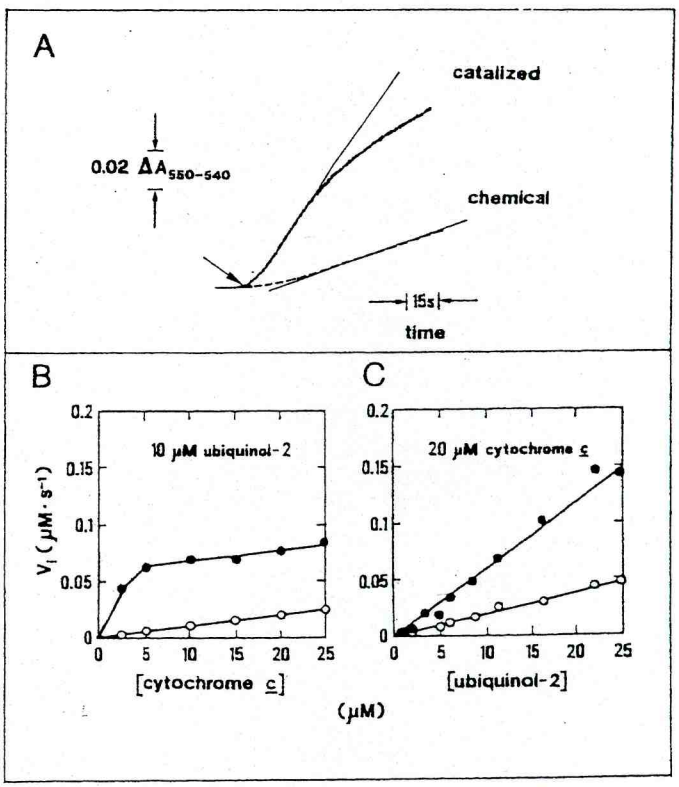

FIG. 2. Steady-state reduction of cytochrome *c* by ubiquinol-2 catalyzed by the Rieske protein. (A) Time course of cytochrome *c* (10 μM) reduction by 18 μM ubiquinol-2 in the absence (---) and in the presence of 0.12 μM Rieske protein added before the quinol (—). The initial rates, extrapolated along the linear portion of the traces, are also shown. (B) Titration of the chemical (○) and catalyzed (●) reaction as a function of cytochrome *c* concentration. (C) Titration of the chemical (○) and catalyzed (●) reaction as a function of ubiquinol-2 concentration. The Rieske protein is 0.11 μM in (A) and 0.1 μM in (B), respectively.

linearly dependent on the concentration of the Rieske cluster above 0.04 μM with any micromolar concentrations of ubiquinol and cytochrome *c* (22).

All the above kinetic features indicate that with 10–20 μM cytochrome *c* and similar concentrations of ubiquinol-2, the steady-state reaction catalyzed by the isolated Rieske iron–sulfur protein fundamentally reflects the oxidation of the quinol in solution.

pH Dependence of Ubiquinol Oxidation and the Role of Oxygen

The pH dependence of the oxidation of ubiquinol-2 catalyzed by the isolated Rieske protein resembles that of the enzymatic activity of the native bc_1 complex. Both reactions display a clearly biphasic behavior of their rates as a function of $[H^+]^{-1}$ concentration, whereas the chemical reaction of ubiquinol-2 with cytochrome *c* follows a linear behavior (Fig. 3), as previously reported for other quinols (10). Such a difference is related to the tendency of reaching a pH optimum around 8 in the catalyzed reactions (see also (23)), presumably as a consequence of ionizable groups that are involved in the rate-limiting steps. In support to this, the rates of ubiquinol-2 oxidation catalyzed by the Rieske protein show a much steeper dependence upon the ionic strength by increasing the pH. For instance, at pH 8 the slope of the ionic strength dependence is two times larger than at pH 6.5 (F. Ballester and J. Timoneda, unpublished results).

Interestingly, the pH dependence of ubiquinol-2 oxidation by the Rieske protein becomes qualitatively identical with that of the chemical oxidation by cytochrome

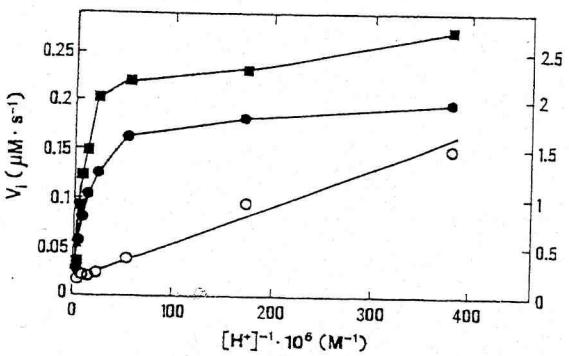

FIG. 3. Comparison of the chemical and catalyzed reduction of cytochrome *c* by ubiquinol-2 as a function of pH. ■, initial rates of the reductase reaction catalyzed by 6 nM bc_1 complex (values on the right of the plot); ●, initial rates of the reaction catalyzed by 0.1 μM Rieske protein; ○, initial rates of the chemical reaction. The concentration of ubiquinol-2 and cytochrome *c* are 18 and 10 μM, respectively. The reactions are measured in the phosphate buffer in the pH range from 6 to 8.6. The data of the catalyzed reactions do not fit a linear dependence upon $[H^+]^{-2}$ (results not shown), thereby indicating that the rates are not determined directly by the semiquinone form (10).

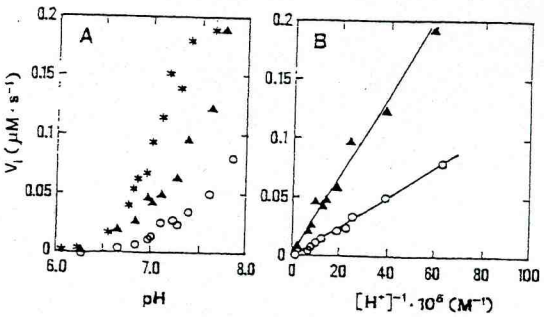

FIG. 4. pH dependence of the chemical and catalyzed reduction of cytochrome *c* by ubiquinol-2 under anaerobic conditions. (A) *, rates of the reaction catalyzed by the Rieske protein under aerobic conditions, i.e., without the addition of glucose oxidase; ▲, rates of the reaction catalyzed by the Rieske protein under anaerobic conditions; ○, chemical reaction under anaerobic conditions. The buffer was 50 mM Tris–acetate, containing 1 mM EDTA, 10 mM glucose, and 40 units/ml of catalase. Anaerobic conditions are achieved by preincubating the assay mixture for 2 h with 10 units/ml of glucose oxidase. The concentrations of the reagents are: 0.12 μM Rieske protein, 13 μM ubiquinol-2, and 9.4 μM cytochrome *c*. (B) Plot of the data in (A) as a function of the inverse of the proton concentration (cf. Fig. 3). The reaction catalyzed by the Rieske protein under aerobic conditions follows a biphasic behavior as in Fig. 3 (results not shown).

c when oxygen is efficiently removed from the medium (Fig. 4; cf. Fig. 3). Under anaerobic conditions, the rates of cytochrome *c* reduction catalyzed by the iron–sulfur protein are slower than under aerobic conditions (Table II), particularly at the physiological pH 7.2–7.4 (Fig. 4A). Moreover, at these pH values, in the absence of oxygen the ionic strength dependence of the catalyzed rates is substantially lower than in the presence of oxygen, whereas it is nearly identical for the chemical rates (results not shown). This suggests that the reaction catalyzed by the Rieske protein involves ionizable groups, including oxygen radicals (see below), which are not involved in the chemical reactions.

Involvement of oxygen radicals in the reduction of cytochrome *c* by ubiquinol-2 is apparently confirmed by the effect of superoxide dismutase, which decreases by approximately one-half both the chemical and the catalyzed rates (Table II). By contrast, the removal of oxygen induces, if anything, a slight increase in the chemical reaction (mostly due to the effect of catalase alone), whereas it halves the reaction catalyzed by the Rieske protein (Table II). Consequently, the effect of superoxide dismutase on the chemical reaction may not be entirely due to the removal of oxygen radicals (10), but also by some catalytic action on semiquinone species. Indeed, concentrations of this enzyme that largely exceed those required for removing the slow formation of superoxides decrease up to 64% the chemical reaction, and still affect the same reaction in the absence of oxygen (Table II). This additional effect of superoxide dismutase on the reaction of ubiquinol-2 with cytochrome *c* may be related

TABLE II

Effect of Various Enzymes and Reagents on the Chemical and Rieske Protein-Catalyzed Reduction of Cytochrome *c* by Ubiquinol-2

Addition	Enzyme concentration[a]	Relative rate[b]
Chemical reaction		
None	—	100
Ubiquinone-2 (8 μM)	—	300[c]
Catalase	125/ml	117
Catalase	45/ml	104
Fe–superoxide dismutase (*E. coli*)	100/ml	51
Cu,Zn–superoxide dismutase	30/ml	50
Cu,Zn–superoxide dismutase	150/ml	46
Cu,Zn–superoxide dismutase	750/ml	36
Cu,Zn–superoxide dismutase plus catalase	90/ml 45/ml	52
Glucose oxidase	30/ml	100[d]
Glucose oxidase plus catalase	10/ml 45/ml	104
Glucose oxidase plus catalase and Cu,Zn–superoxide dismutase	10/ml 45 and 150/ml	90
Catalyzed reaction[e]		
None	—	100
Cu,Zn–superoxide dismutase	150/ml	58
Cu,Zn–superoxide dismutase plus catalase	150/ml 45/ml	50
Glucose oxidase plus catalase	10/ml 45/ml	50

[a] As specified from Sigma purchaser, in units.

[b] The values are the mean of three to seven experiments in the phosphate buffer containing 30 mM glucose with 10–20 μM cytochrome *c* and 15–20 μM ubiquinol-2. Rates are 0.02–0.05 μM s^{-1}.

[c] The same rate is seen under anaerobic conditions.

[d] In the buffer without glucose; in the glucose-containing buffer the time course of cytochrome *c* reduction is bell-shaped and incomplete because of the reoxidizing effect of H_2O_2 on the reduced cytochrome, yet the V_i is very similar to that of the control.

[e] In the presence of variable concentrations of the Rieske protein, generally around 0.1 μM.

to its semiquinone–superoxide oxidoreductase activity that has been recently discovered (24).

The Role of Semiquinone Anion in the Reduction of Cytochrome c

The above effects of oxygen in the reactions between cytochrome *c* and ubiquinol-2 require a more direct assessment of the route of the second electron derived from the oxidation of ubiquinol. This electron should be carried by the semiquinone anion, the radical species prevailing at neutral pH (10–13). We have found a simple way of monitoring ubisemiquinones generated in solution with short-chain ubiquinol homologs by exploiting the aggregation phenomena that occur around their critical micellar concentration (21, 25, 26). The aggregation in the micellar phase prolongs the stability of the semiquinone radicals that are spontaneously formed in solution by ubiquinol disproportionation (27), as seen by the absorbance increase between 400 and 500 nm when the ubiquinol concentration approaches or exceeds its critical self-aggregating value (Fig. 5). The absorbance spectra thus generated, when corrected for the light scat-

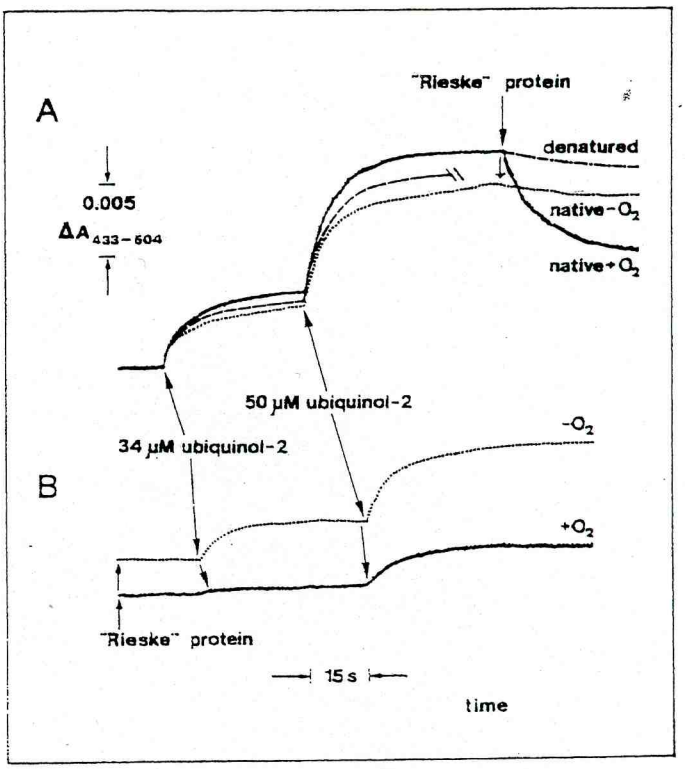

FIG. 5. Detection of ubisemiquinone-2 anion in solution and effects of various agents on its stabilization. The reaction is monitored at 433–504 nm where no spectral interference from the reduction of cytochrome *c* could be detected. The amplitude of the absorbance due to the aggregated semiquinone is twofold smaller, however, than that at 440–600 nm (22). The phosphate buffer, pH 7.2, contains 30 mM glucose; removal of oxygen is accomplished as described in the legend of Fig. 4. The final concentrations of ubiquinol-2 after the additions (arrows) is shown in the figure. The concentration of the Rieske protein is 0.17 μM in all cases. —, ubiquinol-2 in solution alone; on the right of (A) is shown the effect of the addition of the native and denatured (---) Rieske protein. A similar effect is seen with equivalent concentrations of the bc_1 complex (22), whereas the addition of nonionic detergents such as 0.1% Triton QS-30 instantaneously abolishes the signal (results not shown). ---, trace obtained in the presence of 10 μM cytochrome *c*, i.e., concomitant to the chemical reaction. ···, trace obtained under anaerobic conditions ($-O_2$); on the right of (A) the small effect produced by the addition of the native Rieske protein is shown. A nearly superimposable trace is obtained when also cytochrome *c* is present under anaerobic conditions (results not shown). In (B) the traces are obtained without cytochrome *c*; its addition, anyway, produces traces very similar to those shown in the figure.

tering produced by the aggregation, show broad maxima around 410 and 440 nm (results not shown), characteristic of the ubisemiquinone anion in solution (21, 28). This radical species is relatively stable in time within the micellar phase, because agents that dissolve the aggregates, such as nonionic detergents, abolish its absorbance signal (22). Although the physical state of the aggregated semiquinone is likely to prevent its redox reactivity (26), it should be considered that the free form of a (semi)quinone is in rapid equilibrium with its micellar form (21, 25). This implies that any substantial decrease of the steady-state level of the aggregated semiquinone monitored by its absorbance signal reflects a concomitant decrease also of the free form in solution, which can be related to the reaction of the latter with oxygen or cytochrome *c*.

The steady-state concentration of the micellar ubisemiquinone-2 anion essentially is unaffected by cytochrome *c* and is decreased only slightly by removal of oxygen (Fig. 5A). This supports the previous indirect evidence (Table II) that the chemical reaction of cytochrome *c* with ubiquinols may not chiefly proceed via oxygen or semiquinone radicals. On the contrary, the reduction of cytochrome *c* catalyzed by the Rieske protein is substantially due to oxygen radicals derived from semiquinone anions, since this protein promotes the destabilization of the ubisemiquinone-2 anion at the expense of oxygen, as shown in Fig. 5A. Among several proteins tested, only *E. coli* Fe–superoxide dismutase apparently shows a similar effect on the micellar semiquinone, although with much less efficiency than the Rieske protein (M. Degli Esposti, unpublished results). Moreover, in the presence of the Rieske protein, little stabilized semiquinone is formed under aerobic conditions, whereas a much larger amount of it is formed under anaerobic conditions (Fig. 5B). Since these latter effects are seen even at quinol concentrations close to its critical micellar concentration (ca. 40 μM (26)), it is very likely that they derive from changes in the levels of both the micellar and free semiquinone anion in solution.

DISCUSSION

The Mechanism of Ubiquinol Oxidation by the Rieske Protein and Its Relevance to the Enzymatic Activity of the bc_1 Complex

The present study provides an exhaustive characterization of the redox reactions of the isolated Rieske iron–sulfur protein from beef heart (Table I). It also gives direct information on the mechanism of ubiquinol oxidation that is catalyzed by this fundamental component of the *bc* and *bf* complexes. Ubiquinol donates one electron to the reducible iron in the isolated Rieske protein via a collisional reaction having a rate constant of 2.2×10^4 M^{-1} s^{-1} under pre-steady-state conditions (Fig. 1A). Such a reaction can be evaluated also from the steady-state reduction of cytochrome *c* under anaerobic conditions and is linearly dependent upon the $[H^+]^{-1}$ concentration (Fig. 4B). This indicates, in agreement with previous conclusions (6, 7, 13), that the ubiquinol anion is the actual reductant of the oxidized Rieske cluster.

The steady-state reduction by ubiquinol-2 of cytochrome *c* that is catalyzed by the Rieske protein proceeds via two independent second-order processes with the ubiquinol-2 anion and cytochrome *c* (Fig. 1). Therefore, it can be described by the same rate equation introduced by Rich for the trimethylquinol–cytochrome *c* reductase of the bc_1 complex (6, 13). It is to be noted, however, that such an equation cannot be applied to the ubiquinol-2–cytochrome *c* reductase of the native bc_1 complex, because the latter displays clear saturation kinetics for each substrate with any concentration of the other substrate (15, 23).

Apparently, the rate of ubiquinol oxidation is four orders of magnitude slower with the isolated Rieske protein than with the parent enzyme (Table I). However, since the ubiquinol-2 anion is the actual reductant and its pK in solution is 12 (7), the real second-order rate constant of the electron transfer to the Rieske cluster in solution can be calculated to be 2×10^9 M^{-1} s^{-1} according to the formulas of Rich (10) applied to the data of Fig. 4B. This rate is now clearly compatible with the second-order rate constant of ubiquinol-2 oxidation derived from the steady-state activity of the bc_1 complex (Table I). The electron transfer to the Rieske center, therefore, can be considered to have a comparable rate when the protein is isolated or inserted into the enzyme, but in the latter case it is accelerated by fast deprotonation of the ubiquinol bound to other protein structures of the complex. This implies, in agreement with previous suggestions (7), that the catalysis of ubiquinol oxidation by the bc_1 complex is basically achieved through a shift from 12 to ca. 8 of the pK for the single deprotonation of ubiquinol. The binding to cytochrome *b* could be largely responsible for this large pK shift.

The Implications of the Interaction between Ubisemiquinone and Oxygen Mediated by the Rieske Protein

There appears to be one important difference between the mode of ubiquinol oxidation by cytochrome *c* and that by the isolated Rieske protein. The former reaction is unaffected, whereas the latter is halved by the removal of oxygen (Table II). It has been previously concluded, basically from the effect of superoxide dismutase (10), that the reaction between quinols and cytochrome *c* proceeds by one-half of its rate via superoxide anions generated by rapid reaction of the semiquinones with molecular oxygen (10, 12). However, the rates of reaction between ubisemiquinones and oxygen are in the range of 10^1 M^{-1} s^{-1} and the overall rate of autoxidation of ubiqui-

nol-2 is $2 \times M^{-1} s^{-1}$ (27), much slower than the observed rate of reaction with cytochrome *c* ($1 \times 10^2 M^{-1} s^{-1}$; Table I and Fig. 2C). Thus, it appears that oxygen radicals derived from reaction with semiquinone could marginally sustain the reduction of cytochrome *c*.

The alternative possibility that the semiquinone reacts with oxygen or, under anaerobic conditions, directly with cytochrome *c* is excluded on the basis of the dependence of the rates upon $[H^+]^{-1}$ (Fig. 4B) instead of the expected dependence upon $[H^+]^{-2}$ that is characteristic of semiquinone-dependent reactions (10). Moreover, the level of the semiquinone anion is not substantially affected by the presence of cytochrome *c* under either aerobic or anaerobic conditions (Fig. 5). Hence, we conclude that the observed inhibitory effect of superoxide dismutase on the chemical reaction is largely due to a direct catalytic action on semiquinones, presumably of the kind described recently (24). This activity would affect the observed rates because it removes the semiquinones that, through rapid dismutation, reform the real reductant of cytochrome *c*, the quinol anion (10–13). Indeed, the second electron from ubiquinol-2 is likely to be recycled through dismutation of its semiquinone forms, as suggested by the autocatalytic stimulation of the chemical reaction by oxidized ubiquinol-2 (Table II). It is to be noted, however, that even if the reaction is autocatalytically stimulated, its rate is dependent upon the $[H^+]^{-1}$ (Figs. 3 and 4) and decreases by increasing the ionic strength (22), thereby implying that the quinol anion is the rate-determining form (10–13).

On the contrary, the reduction of cytochrome *c* by ubiquinol in the presence of the Rieske protein does involve superoxide anions since: (i) removal of oxygen reduces the observed rate by one-half (Table II); (ii) the steady-state concentration of the ubisemiquinone-2 (stable) anion is depressed by the Rieske protein only when oxygen is present (Fig. 5); (iii) the Rieske protein enhances the consumption of oxygen by ubiquinol-2 in solution (results not shown). The above implies that the Rieske protein mediates electron transfer from semiquinone anion to oxygen, the subsequent reaction between superoxide radicals and cytochrome *c* not being rate-limiting under the conditions employed here (rate constant ca. 1×10^5 $M^{-1} s^{-1}$ (29)). Moreover, such an action may be related to the catalytic function of the parent bc_1 complex, as suggested by the similar pH dependence under aerobic conditions (Fig. 3).

Explanations for this semiquinone–oxygen redox activity of the Rieske protein could reside in the peculiar structural properties of its cluster. Unlike in the typical 2Fe2S proteins, the irons in the Rieske protein are coordinated also to histidines (17, 30–34). Structural–functional correlations could be made, therefore, between the Rieske protein and other iron proteins that have histidine ligands and react with both oxygen and semiquinones, such as protocathecuate dioxygenase (35). Indeed, the Fe–superoxide dismutase of *E. coli*, whose structure resembles that of protocathecuate dioxygenase (35, 36), is capable of destabilizing the micellar ubisemiquinones like the Rieske protein.

Finally, there is an interesting physiological implication of the semiquinone–oxygen reaction mediated by the Rieske protein. Electron transfer along the mitochondrial respiratory chain is known to generate oxygen radicals at the level of the bc_1 complex (27, 37). The formation of such radicals is enhanced by antimycin (27, 37–39) and completely abolished by center "o" inhibitors (37, 38). Given that these inhibitors block ubiquinol oxidation by the Rieske cluster (6, 7), it is likely that this reaction generates oxygen radicals (14), particularly in "slipping" turnovers of the bc_1 complex. The Rieske protein, because of its intrinsic property of diverting the electrons from ubisemiquinone to oxygen (see above), may therefore be a major generator of the oxygen radicals produced during mitochondrial respiration. This contrasts with common views that consider ubisemiquinone or cytochrome *b* the direct generator of oxygen radicals at the second coupling site in mitochondria (28, 37, 38).

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