THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 269, No. 49, Issue of December 9, pp. 31006–31011, 1994
Printed in U.S.A.

Sulfolobus acidocaldarius Terminal Oxidase

A KINETIC INVESTIGATION AND ITS STRUCTURAL INTERPRETATION*

(Received for publication, May 5, 1994, and in revised form, October 3, 1994)

Alessandro Giuffrè, Giovanni Antonini, Maurizio Brunori‡, Emilio D'Itri, Francesco Malatesta, Flavia Nicoletti, Stefan Anemüller§, Michael Gleissner§, and Günter Schäfer§

From the Departments of Biochemical Sciences, Universities of Rome "La Sapienza" and "Tor Vergata" and CNR Center of Molecular Biology, Rome, Italy, and the §Institute of Biochemistry, Medical University of Lübeck, Lübeck, Germany

The thermoacidophilic archaebacterium *Sulfolobus acidocaldarius* possesses a very unusual terminal oxidase. We report original kinetic experiments on membranes of this microorganism carried out by stopped flow, using time-resolved optical spectroscopy combined with singular value decomposition analysis. The reduced-oxidized kinetic difference spectrum of the *Sulfolobus* membranes is characterized by three significant peaks in the visible region at 605, 586, and 560 nm. The 605-nm peak and part of the 586-nm peak (cytochrome aa_3-type quinol oxidase) are reduced synchronously by both ascorbate plus *N,N,N′,N′*-tetramethyl-*p*-phenylendiamine (TMPD) and dithionite, and they are very rapidly oxidized by molecular oxygen. A second pool of cytochromes seems to contribute to the 586-nm peak which is not reduced by ascorbate plus TMPD and reacts very slowly with dithionite. The *b*-type cytochromes (560 nm peak) are reduced by both reductants and are essentially "non-autoxidizable" at room temperature. Only one CO binding site with spectral features, kinetic properties, and ligand affinity not very dissimilar from those of mammalian cytochrome oxidase can be detected in the ascorbate-reduced membranes. On the contrary, a second CO binding site having unusual properties for aa_3 terminal oxidases can be detected in the dithionite-reduced membranes.

The archaebacterium *Sulfolobus acidocaldarius* grows at temperatures around 85 °C in acidic media (pH 2–3). It is a strict aerobic microorganism reducing oxygen to water to sustain across its plasma membrane a large proton gradient which is the driving force for ATP synthesis via an F_0F_1-like ATP-synthase (1). The respiratory chain of this extremophile is quite unusual, since *c*-type cytochromes are totally absent, and caldariellaquinone (2) plays a key role, accepting electrons from dehydrogenases previously characterized (3, 4). Several *a*- and *b*-type cytochromes have been identified in *S. acidocaldarius* membranes. Some of the *a*-type cytochromes belong to an aa_3 terminal oxidase, which uses caldariellaquinol as electron donor and oxygen as electron acceptor. This terminal oxidase can be purified as a "single subunit" (5) or a multisubunit (6) enzyme, and depending on subunit composition, it displays distinct spectroscopic features and heme content (see below). The physiological role of the *b*-type cytochromes, which have been purified and partially characterized (7), as well as that of a Rieske-type Fe-S, discovered by Anemüller *et al.* (8), are essentially unknown.

The reduced-oxidized absorption spectrum of *S. acidocaldarius* membranes shows in the visible region three major bands centered at 605, 586 and 560 nm. The 605-nm band was assigned to the aa_3-type terminal oxidase, on the basis of observations on the purified single subunit enzyme; with the purified multisubunit complex, additional *a*-type hemes were detected with an unusually intense absorption band centered at 586 nm. The 560-nm band was assigned to *b*-type cytochromes, and low temperature spectra revealed that this band is composed by more than one optical component (7).

In the present study we report results obtained by a combination of transient optical spectroscopy (with a time resolution of 5 ms) and singular value decomposition analysis on native *S. acidocaldarius* membranes. The kinetic analysis of the optical changes due to the different chromophores during the reaction of the microbial membranes with ascorbate plus TMPD,[1] dithionite, oxygen, and carbon monoxide strongly supports the hypothesis that the aa_3-quinol-oxidase is the prevailing oxygen-reducing enzyme in *S. acidocaldarius* membranes. The kinetic and optical data are discussed with reference to a possible structural model (6) of this enzymatic complex, and surprisingly they were found fairly consistent, providing further information for an understanding of this interesting terminal oxidase.

EXPERIMENTAL PROCEDURES

Materials—Membranes from *S. acidocaldarius*, prepared according to Anemüller and Schäfer (5), were resuspended in 50 mM malonate + 1 mM EDTA, pH 5.5, at a total protein concentration of 22 mg/ml and stored at −70 °C. Membranes were diluted 7-fold in different buffers (see figure legends) and sonicated on ice for 1 min before use. The aa_3-quinol-oxidase concentration was estimated from the reduced-oxidized spectrum using the extinction coefficient $\Delta\epsilon(605) = 22\ mM^{-1}\ cm^{-1}$ (9).

Ascorbate, TMPD, and Sarcosyl were from Sigma.

Spectrophotometry—CO titrations were monitored by double beam spectrophotometry on an OLIS-Cary 14, after membrane solubilization with 1.5% Sarcosyl in order to reduce light scattering in the Soret region. A 1 mM CO stock solution was prepared equilibrating degassed buffer under CO atmosphere at 20 °C. Titrations were carried out on 1-cm light path sealed cuvettes, taking care of reducing as much as possible the gas phase trapped in the cuvettes. Spectra were collected at room temperature.

* This work was partially supported by Ministero Dell' Universita' E Della Ricerca Scientifica E Tecnologica of Italy (40% Liveprotein), by the European Union G-project on Biotechnology of Extremophiles no. PL 920274 (Associated Contractor no. 26), and by the Deutsche Forschungsgemeinschaft, Scha 125/17-2 (to G. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dipartimento di Scienze Biochimiche "A. Rossi Fanelli," Universita' di Roma "La Sapienza," P. le Aldo Moro 5, 00185 Roma, Italia. Tel.: 39-6-4450291; Fax: 39-6-4440062.

[1] The abbreviations used are: TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylendiamine; SVD, singular value decomposition.

Time-resolved Optical Spectroscopy—Time-resolved spectra were collected with a photodiode array spectrophotometer (TN6500, Tracor Northern, Madison, WI) adapted to a thermostated Durrum-Gibson stopped-flow apparatus with a 2-cm light path. This rapid scanning spectrophotometer acquires 512 or 1024 diode elements in 5 or 10 ms, and up to 80 spectra can be recorded as a function of time.

Data Analysis—Data analysis was carried out by means of the software MATLAB (MathWorks, South Natick, MA) running on an Intel 486 based computer. Spectral smoothing and optical deconvolution were performed by using singular value decomposition (SVD, see Ref. 10). A matrix of time-resolved spectra (A) is decomposed by SVD into the product of three matrices, $A = U \times S \times V^T$, where U and V are orthogonal, and S is a diagonal matrix. The columns of the U matrix yield the basis spectra of the decomposition which are ordered in terms of their decreasing occupancies in the original data matrix. The time dependencies of these basis spectra (U columns) are represented by the columns of the V matrix, while the diagonal values of the S matrix, so called "singular values" (all non-negative), yield the relative occupancies of the basis spectra within the data set. An important property of the SVD is that signal and noise content of a data set are partially split along the U and V columns so that random noise is mainly accumulated in the last U and V columns, those with lower occupancies. Therefore reconstruction of the original data set with higher signal-to-noise ratio can be performed with a subset of basis spectra, choosing those with higher occupancies, and their associated S values and V columns. If a data set is contributed by more than one optical transition, deconvolution of the optical components, provided they have different time courses, can be achieved by simultaneously fitting the chosen V columns subset to the desired kinetic scheme; the resulting amplitudes matrix can be used to reconstruct the optical species from the chosen subset of basis spectra.

RESULTS AND DISCUSSION

Reaction with Dithionite—S. acidocaldarius membranes were mixed anaerobically in the photodiode array stopped-flow spectrophotometer against dithionite at 20 °C. The time recording mode was logarithmic to properly cover fast and slow processes occurring in the chosen time domain (from 5 ms to approximately 300 s).

Analysis of spectra such as those depicted in Fig. 1 (*Panel a*) was carried out by SVD; the first three U and V columns of the SVD output (Fig. 1, *Panels b–e*) clearly indicate a complex spectral and kinetic behavior. Deconvolution of the optical components was achieved by simultaneously fitting the time dependence of these three V columns to the simplest kinetic scheme involving two parallel first-order processes. This possible mechanism has been substantiated by experiments carried out on the purified three-subunit enzyme (6).[2] The fitted rate constants for the fast and slow phase were $k_1 = 3.3\ s^{-1}$ and $k_2 = 0.07\ s^{-1}$, respectively, and their optical difference spectra after deconvolution are also shown in Fig. 1 (*Panels f* and *g*). Spectral deconvolution clearly indicates that part of the 586-nm band is reduced by dithionite slowly, whereas the remaining fraction of the 586-nm band is reduced more rapidly and synchronously with the 605-nm band. Additional experiments showed that the faster process is dithionite concentration-dependent (data not shown).

These results suggest that the biphasic kinetics observed in the reaction with dithionite is largely due to heterogeneity in the 586-nm band, which is therefore assigned to (at least) two different species with different reactivity toward dithionite. A significant fraction (>50%) of the 586-nm cytochromes is indeed reduced very slowly by dithionite, whereas all of the other chromophores are reduced more rapidly and with approximately the same rate constant.

Reaction with Ascorbate plus TMPD—Respiration of *Sulfolobus* membranes can be sustained by ascorbate as electron donor, and it is stimulated severalfold by addition of TMPD (data not shown), which is consistent with the finding that reduced

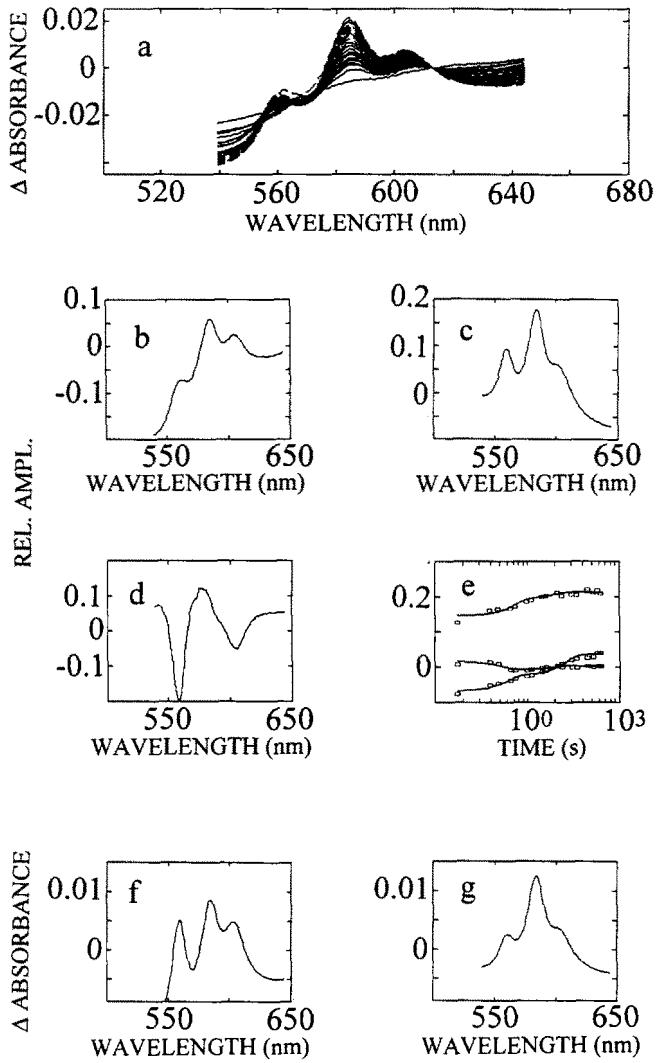

FIG. 1. **Reduction by dithionite.** Membranes diluted in 200 mM potassium phosphate pH 7 (total protein concentration approximately 3 mg/ml) were degassed and mixed anaerobically at 20 °C in the diode-array stopped-flow apparatus against N_2-equilibrated 20 mM dithionite in the same buffer. *a*, spectra acquired in the visible region during the onset of reduction (base line: oxidized membranes). This spectra set was analyzed by SVD. *b*, *c*, and *d*, first three U columns of the SVD output with relative singular values ($S_1 = 1.8$; $S_2 = 0.31$; $S_3 = 0.05$). *e*, best fit (*solid lines*) of the first three V columns scaled by their relative singular values (*open cirles*) according to two parallel first-order decays. Fitted rate constants are $k_1 = 3.3\ s^{-1}$ and $k_2 = 0.07\ s^{-1}$. *f* and *g*, fast and slow optical components deconvoluted from the fit above. Note that the slow phase is largely due to a chromophore absorbing at 586 nm.

TMPD can serve as an effective electron donor for the aa_3-type oxidase (5).

The reaction of *Sulfolobus* membranes with ascorbate plus TMPD was investigated by rapidly mixing degassed membranes against air-equilibrated ascorbate plus TMPD at 20 °C. These experiments revealed that *a*-type and *b*-type cytochromes are characterized by clearly different kinetic behavior (Fig. 2). Starting with fully oxidized membranes, the reduction level of the *b*-type cytochromes increases biphasically as the reaction proceeds, as shown by the time courses at 560 nm; a rather fast reaction of about 30% may correspond to cytochrome *b*-558 which is accessible to reduction by ascorbate even in the presence of oxygen. On the contrary, for *a*-type cytochromes (605- and 586-nm peaks) a steady-state level is rapidly reached and maintained (for ≈50 s) in so far as oxygen is present (Fig. 2); after O_2 exhaustion, the *a*- and *b*-type cyto-

[2] A. Giuffrè, G. Antonini, M. Brunori, E. D'Itri, F. Malatesta, F. Nicoletti, S. Anemüller, M. Gleissner, and G. Schäfer, unpublished results.

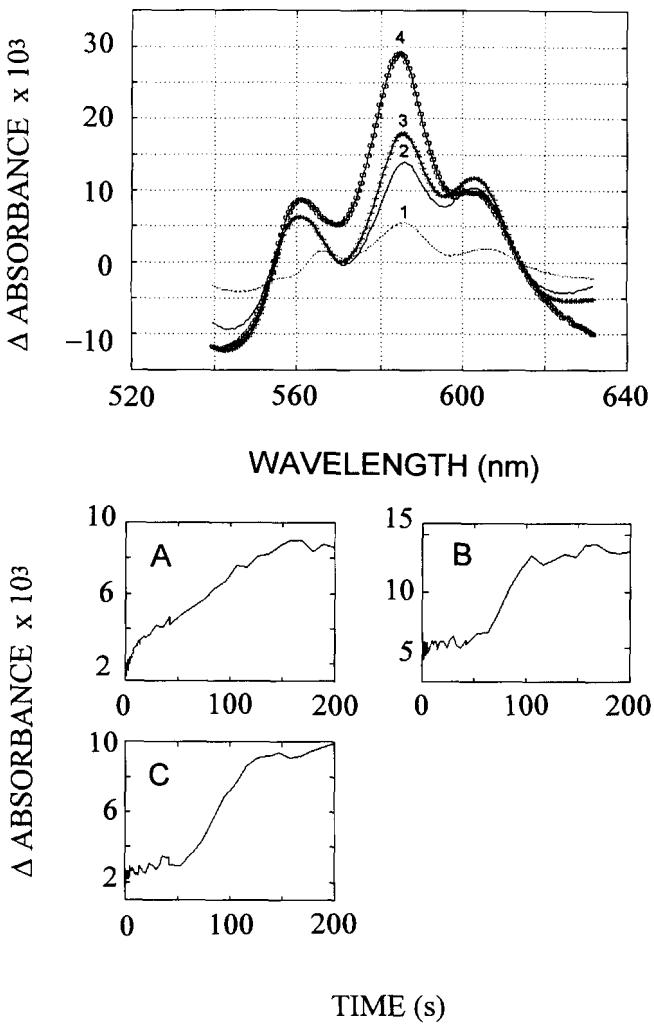

FIG. 2. **Reaction with ascorbate and TMPD.** Membranes diluted in 200 mM potassium phosphate, pH 7.2 (total protein concentration approximately 3 mg/ml) were degassed and rapidly mixed against air-equilibrated 10 mM ascorbate and 20 μM TMPD in the same buffer at 20 °C. Base line: oxidized membranes. (*Top panel*) *1*, spectrum recorded during the steady-state phase; *2*, spectrum acquired immediately after oxygen exhaustion and subsequent re-reduction of *a*-type cytochromes by the excess reductant. *3*, spectrum recorded 15 min after oxygen exhaustion. *4*, spectrum of dithionite-reduced membranes. (*Bottom panels*) Time courses recorded at: *A*, 560–553 nm (*b*-type cytochromes); *B*, 586–571 nm (*a*-586); *C*, 605–614 nm (aa_3). Note the steady-state phase (50 s) of the *a*-type cytochromes (in *B* and *C*), and compare with the time course of the *b*-type cytochromes reduction (in *A*).

chromes are all reduced by the excess reductant. At this time, the total absorption increase observed at 560 and 605 nm is similar in amplitude to those recorded at the same wavelengths upon reduction by dithionite (see Fig. 2, *top panel*). On the contrary the amplitude of the 586 nm band is definitely lower with respect to the dithionite-reduced membranes (and close to that recorded during the fast phase of the reaction with dithionite); even spectra collected 15 min after O_2 exhaustion do not reach the extent of reduction achieved by dithionite (Fig. 2).

No significant difference in amplitude or in kinetics was observed at low and high ionic strength, whereas a strong temperature dependence of the reaction was ascertained by varying temperature from 10 to 40 °C (data not shown).

These results show that (at least) two different cytochrome species contribute to the absorbance change at 586 nm, one only efficiently reduced by ascorbate plus TMPD. The steady-state kinetic behavior of the *a*-type cytochromes is consistent with the fact that the 605-nm band and part of the 586-nm band are spectroscopic signatures of a multichromophore oxidase, in agreement with Lübben *et al.* (6). Moreover, the results indicate that if electron transfer from *b*-type cytochromes to O_2 occurs via the aa_3-type terminal oxidase, under these experimental conditions it is not competitive with the ascorbate plus TMPD pathway; this is shown (Fig. 2) by the observation that the time course at 560 nm does not follow the steady-state kinetics observed for *a*-type cytochromes (time courses at 586 and 605 nm). It may be pointed out that with our present instrument the 560-nm band behaves as a single spectral component (with no sign of the three peaks resolved at low temperature; see Ref. 7).

Reaction with Oxygen—N_2-equilibrated membranes were reduced by ascorbate and TMPD and rapidly mixed at 20 °C against buffer containing approximately 30 μM O_2. The rationale of this kind of experiments (so called O_2 pulse experiment) is that oxidation of reduced terminal oxidases by O_2 is usually so fast (11) to be completed within the dead-time of the stopped-flow apparatus (3 ms). Therefore, a spectrum collected at $t = 0$ should have the spectroscopic features of fully oxidized oxidase, which is thereby (partially) re-reduced by ascorbate plus TMPD to a steady-state level eventually maintained until O_2 is exhausted. On the other hand, other components of the respiratory chain which are not reactive toward O_2 will be and stay reduced throughout.

The averaged spectrum recorded between 5 and 50 ms after mixing is reported in Fig. 3 (*spectrum 1*) together with the spectrum acquired after O_2 exhaustion (*spectrum 3*), when the excess reductant brings all the cytochromes to complete reduction. As shown in Fig. 3, the only significant absorbance changes observed involve the 586- and 605-nm bands (aa_3-quinol-oxidase), while no synchronous oxidation of the *b*-type cytochromes (560 nm) can be observed on this time scale. The time courses depicted in Fig. 3 illustrate the steady-state behavior of *a*-type cytochromes and reinforce the conclusion that at this temperature (20 °C) the *b*-type cytochromes are *very slowly* oxidized by O_2, and very slowly re-reduced when this is totally consumed. Experiments carried out at higher temperatures (up to 40 °C) revealed that the *a*-type cytochromes steady-state duration is much shorter, while the *b*-type cytochromes oxidation is slightly enhanced.

Another relevant observation (also shown in Fig. 3) is that, if TMPD concentration is increased from 2 to 20 μM before mixing, the steady-state phase of both 586- and 605-nm bands is characterized by higher reduction level (Fig. 3, *spectrum 2*) and shorter duration (data not shown). Again, this result is consistent with the finding that reduced TMPD is an effective electron donor for the aa_3-type oxidase (5) and indicates that in the experiment previously described ([TMPD] = 2 μM, before mixing) electron entry into the oxidase was rate-limiting.

These results taken together clearly indicate that reduced *a*-type cytochromes are very rapidly oxidized by O_2 (and then re-reduced by ascorbate plus TMPD), whereas the *b*-type cytochromes are not oxidized by O_2 at a significant rate. Therefore it is independently confirmed that the *Sulfolobus* oxidase is represented by the chromophores absorbing at 605 nm (aa_3-Cu_B) as well as some of the species absorbing at 586 nm (also heme *a*). As a tentative corollary of these results it seems that the *b*-type cytochromes (560 nm) are in slow redox equilibrium with the aa_3-type oxidase; this hypothesis being correct, it cannot be excluded that the efficiency of electron transfer through this pathway may be enhanced at physiological temperatures.

CO Binding Kinetics—CO binding reactivity was investigated by anaerobically mixing in the photodiode-array stopped-flow apparatus dithionite-reduced membranes against CO in potassium phosphate. Several experiments were carried out at

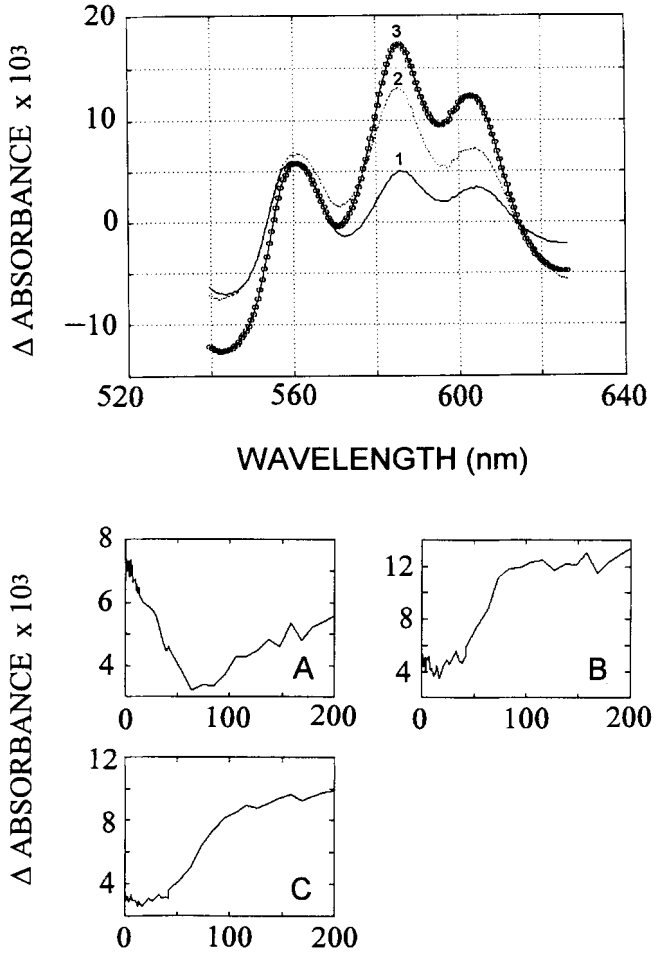

FIG. 3. **O_2 pulse experiment.** Membranes diluted in 20 mM potassium phosphate, pH 7.2 (total protein concentration approximately 3 mg/ml), were degassed, reduced by 5 mM ascorbate and 2 μM TMPD, and rapidly mixed at 20 °C against the same buffer containing oxygen at approximately 30 μM. Base line: oxidized membranes. (*Top panel*) *1*, average of the spectra collected up to 50 ms after mixing indicates that the 586- and 605-nm bands have an absorbance significantly smaller than that of spectrum 3 collected after O_2 exhaustion; this indicates (see text) that the only cytochromes rapidly oxidized by oxygen are those belonging to the aa_3-quinol-oxidase. *2*, the same as in *1*, but in the presence of 20 μM TMPD. The higher steady-state reduction level is consistent with the evidence that TMPD is an effective electron donor for aa_3-quinol-oxidase and proves that, in *1*, electron entry into the oxidase was rate-limiting. *3*, spectrum acquired immediately after oxygen exhaustion and complete re-reduction of the system by the excess reductant. (*Bottom panels*) time courses recorded at: *A*, 560–573 nm (*b*-type cytochromes); *B*, 586 nm (*a*-586); *C*, 605 nm (aa_3). Compare the steady-state kinetic behavior of the *a*-type cytochromes (in *B* and *C*) with the time course of the *b*-type cytochromes (in *A*).

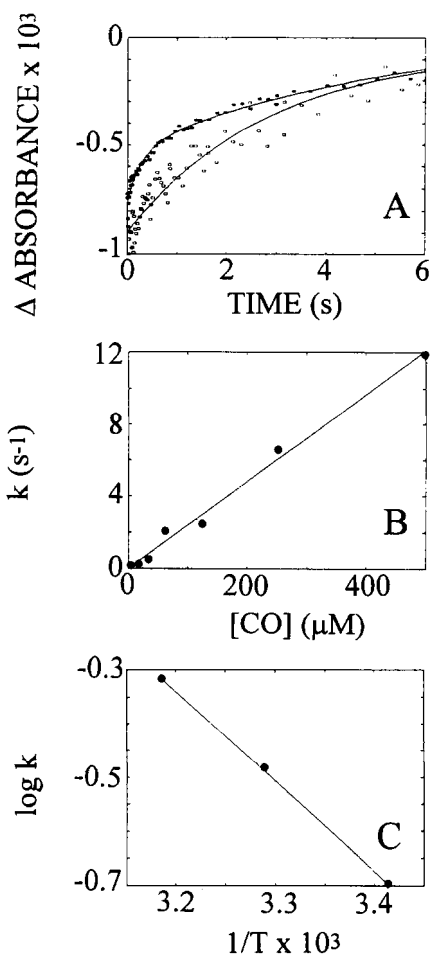

FIG. 4. **CO binding kinetics.** *Panel A*, time courses at 599 nm of CO binding to dithionite-reduced (*full circles*) and ascorbate plus TMPD-reduced (*open circles*) membranes, reconstructed after SVD signal filtering. *Solid lines* are best fits to a two exponentials and a one exponential decay, respectively. Temperature = 20 °C; total protein concentration, approximately 3 mg/ml. *Full circles*, time course observed on mixing dithionite-reduced membranes in 100 mM potassium phosphate, pH 7, against 30 μM CO. k_1 = 3.5 s^{-1}, k_2 = 0.18 s^{-1}. *Open circles*, time course observed on mixing membranes in 20 mM potassium phosphate, pH 7, previously reduced by 5 mM ascorbate and 10 μM TMPD, against 100 μM CO plus a small amount of dithionite. k' = 0.34 s^{-1}. *Panel B*, dependence on CO concentration of the pseudo-first-order rate constant for the slow phase of CO binding to dithionite-reduced membranes at 20 °C (see *Panel A*). The bimolecular rate constant estimated from the regression line is $k = 2.5 \times 10^4$ M^{-1} s^{-1}. *Panel C*, Arrhenius plot of CO binding to the slow species in dithionite-reduced membranes. Estimated activation energy of the reaction is E^* = 7.6 kcal mol^{-1}.

different CO concentrations (from 15 μM to 1 mM before mixing) and temperatures (20, 30, and 40 °C) in order to estimate the apparent bimolecular rate constant and the activation energy of the reaction.

The observed signals were characterized by small amplitudes (~10^{-3} absorbance units), and their analysis was complicated by base-line shifts and random noise; nevertheless, after signal filtering by averaging and SVD analysis, single wavelength time courses were reconstructed from the SVD output and showed biphasic behavior (Fig. 4, *Panel A*). Time courses were fitted by a least-square algorithm to two parallel exponential decays; a typical time course recontructed at 599 nm and its best fit are shown in Fig. 4 (*Panel A*). Pseudo-first-order rate constants calculated for the slow phase were found to be linearly dependent on CO concentration (Fig. 4, *Panel B*) and the apparent bimolecular rate constant value was estimated as $k = 2.5 \times 10^4$ M^{-1} s^{-1}. From the temperature dependence of the rate constant (Fig. 4, *Panel C*), the activation energy of the slower process has been estimated at E^* = 7.6 kcal mol^{-1}. Interestingly the bimolecular rate constant and the activation energy are both fairly similar to those reported for CO binding to reduced bovine cytochrome *c* oxidase, *i.e.* $k = 8 \times 10^4$ M^{-1} s^{-1} and E^* = 6.4 kcal $mole^{-1}$ (11).

In one experiment the kinetics of CO binding was investigated by mixing at 20 °C membranes previously degassed and reduced only by ascorbate plus TMPD. Data collection and analysis were performed as described above. Unlike the dithionite-reduced membranes, the time course was monophasic (Fig. 4, *Panel A*) and fit to a single exponential process yielded an

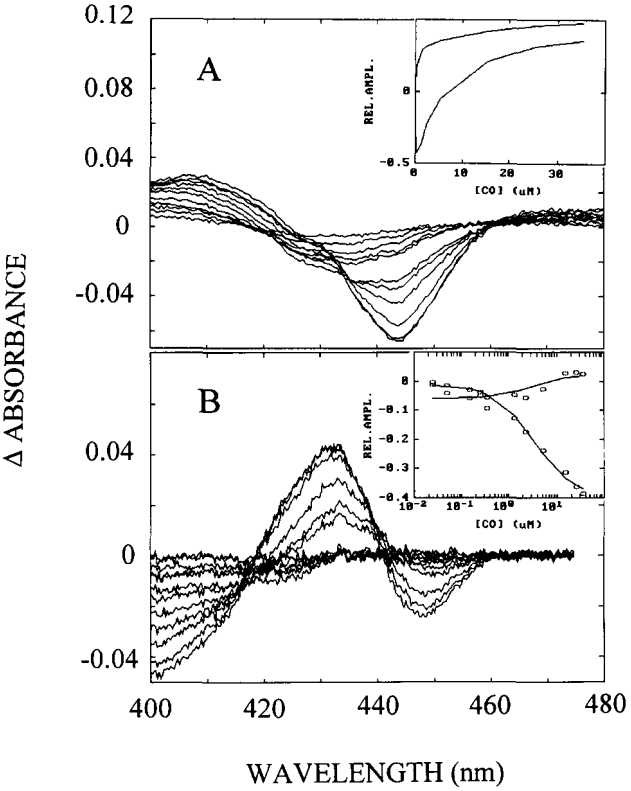

FIG. 5. **CO titrations.** CO titration at room temperature of dithionite-reduced (*Panel A*) and ascorbate plus TMPD-reduced (*Panel B*) membranes, previously solubilized by 1.5% Sarcosyl and degassed. Light path = 1 cm. Increasing CO concentrations: 25 nM, 50 nM, 150 nM, 250 nM, 350 nM, 1.3 μM, 2.3 μM, 5.3 μM, 15.3 μM, 35.3 μM. Total protein concentration, approximately 3 mg/ml. *Panel A*, difference spectra obtained titrating dithionite-reduced membranes with CO, using the reduced sample as reference. Buffer: 10 mM Hepes, pH 7. *Inset*, first two *V* columns of the SVD output showing that CO binding is biphasic. *Panel B*, difference spectra obtained titrating ascorbate plus TMPD-reduced membranes with CO, using the reduced sample as reference. Buffer: 50 mM potassium phosphate, pH 7. Ascorbate, 5 mM; TMPD, 50 μM. *Inset*, best fit (*solid line*) of the first two *V* columns (*open circles*) of the SVD output to a single binding site model. The fitted affinity constant ($K_a = 3 \times 10^5$ M^{-1}) was calculated for an oxidase concentration of 0.8 μM functional unit (from the reduced-oxidized difference spectrum, as described under "Experimental Procedures"). Varying arbitrarily the oxidase concentration from 0.1 to 2.5 μM made no change to the value of K_a.

estimated bimolecular rate constant ($k = 0.7 \times 10^4$ M^{-1} s^{-1}), smaller but within the same order of magnitude as that observed for the slow phase of CO binding to dithionite-reduced membranes.

CO Titration—To rationalize kinetic differences in CO binding to dithionite- and ascorbate plus TMPD-reduced membranes, static CO titrations were carried out on membranes solubilized by Sarcosyl; observed difference spectra are reported in Fig. 5. CO titration of the dithionite-reduced membranes (Fig. 5, *Panel A*) is characterized by two optical components with significantly different ligand affinities; this is clearly indicated by comparison of the first two *V* columns of the SVD output, showing definitely different titration profiles (Fig. 5, *Panel A*, *inset*). On the other hand, in the case of ascorbate plus TMPD-reduced membranes, only one optical transition occurs (Fig. 5, *Panel B*); the affinity constant for the latter titration was estimated as $K_a = 3 \times 10^5$ M^{-1}, by fitting the first two *V* columns of the SVD output (Fig. 5, *Panel B*, *inset*) to a single binding site. This affinity constant is different from that reported for bovine cytochrome oxidase (11), *i.e.* $K_a = 3 \times 10^6$ M^{-1}; the difference spectrum CO bound-reduced enzyme reconstructed from this titration is on the other hand similar to that

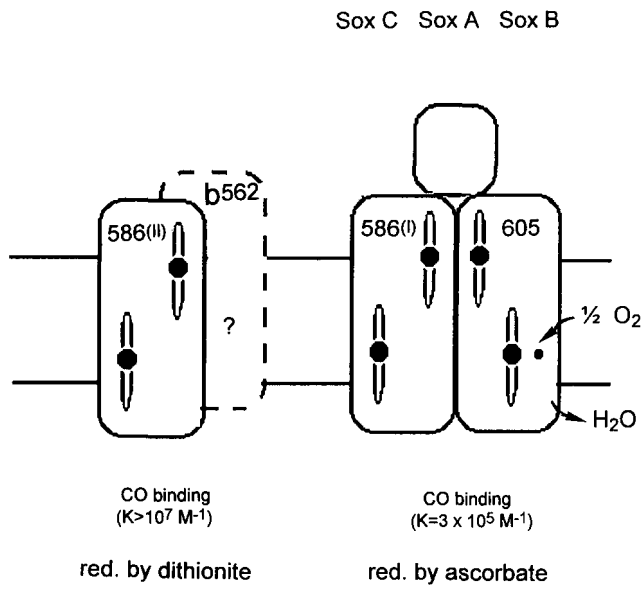

FIG. 6. **Schematic model of the *a*-type cytochromes of *S. acidocaldarius*.** According to Lübben *et al.* (6), the aa_3-quinol-oxidase is a three subunit enzyme (coded by the *SoxA*, *SoxB*, and *SoxC* genes), with five redox centers. SoxB phenotype contains two *a*-type hemes (only one forming a binuclear center with Cu_B) with spectroscopic features very similar to canonical terminal oxidases; two additional *a*-type hemes, absorbing at 586 nm, are bound to the SoxC phenotype. A second pool of *a*-586 cytochromes only reduced by dithionite is related to the high affinity CO binding site.

of the mammalian enzyme, but displays a 3-nm blue shift (data not shown).

These results together with those described in the previous section suggest that CO binding to the aa_3-type oxidase in *Sulfolobus* membranes has kinetic properties, ligand affinity, and spectral features resembling those of the bovine enzyme. On the other hand, the results obtained with the dithionite-reduced membranes indicate that an additional cytochrome, capable of binding CO with very high affinity, is observed only when a strong reductant (such as dithionite) is used. Comparison between the reduced-oxidized difference spectra (Fig. 2) obtained with dithionite- or ascorbate plus TMPD-reduced membranes provides an indication (but does not prove) that the latter CO binding species could be one of the *a*-type cytochromes absorbing at 586 nm. A possible interpretation is that this cytochrome is not reduced by ascorbate plus TMPD due to very low redox potential, but we can not exclude that this CO binding species is only detectable in the dithionite-reduced membranes as a result of some artifact related to the presence of dithionite or by-products of dithionite oxidation.

CONCLUDING REMARKS

We wish to discuss the kinetic data reported above with reference to a possible scheme of the structure and assembly of *S. acidocaldarius* *a*-type cytochromes, as shown in Fig. 6. Based on the structure of the operon coding for the aa_3-quinol-oxidase, the latter is reported to be a three-subunit enzyme (coded by the *SoxA*, *SoxB*, and *SoxC* genes) containing five redox centers, *i.e.* four *a*-type hemes and 1 copper, and forming a complex that has been purified (6). The *SoxB* gene encodes for a protein equivalent to part of cytochrome-*c*-oxidase, having a binuclear center (cytochrome a_3-Cu_B) where the O_2 reaction should take place, and a cytochrome *a* presumably as electron-accepting site. This protein has been purified from the rest of the complex and displays spectroscopic features typical of a terminal oxidase (5); redox titrations followed by EPR spectroscopy (12) have led to the characterization of a low potential and

a high potential *a*-type heme. The electron donor is a quinol (caldariellaquinol) and not cytochrome *c* as in the mitochondrial enzyme; nevertheless it is a *bona fide* terminal oxidase. Two additional *a*-type hemes found in the complex (Fig. 6) are bound to the protein encoded by the *SoxC* gene and are partially responsible for the absorption band at 586 nm. As we shall see below, most of the kinetic results described above are quite consistent with this model, which therefore gains strength from interpretation of the kinetic data on *S. acidocaldarius* membranes.

Relevant information on the respiratory enzymes of *S. acidocaldarius* was obtained by investigating the reaction of membranes reduced by ascorbate plus TMPD with O_2 and the subsequent re-reduction (as illustrated in Fig. 3). These O_2 pulse experiments reveal that the *b*-type cytochromes (with absorption at 560 nm) are *very slowly* oxidized by O_2, indicating that they are not active components of an oxidase. Moreover, these *b*-type cytochromes, which (directly or via a redox shuttle) may be electron donors of the quinol oxidase, under our experimental conditions are bypassed by the electron transfer pathway sustained by ascorbate plus TMPD. This conclusion is supported by the finding that in these experiments (as well as in the reduction of the membranes by ascorbate plus TMPD) the time course of the *b*-type cytochromes is totally different in all phases from that of the *a*-type cytochromes (Fig. 3). In these O_2 pulse experiments spectra collected immediately (5–50 ms) after mixing clearly indicate that the only sites which are rapidly autoxidizable are those bound to the phenotypes of *SoxB* (aa_3-605) and *SoxC* (*a*-586). Moreover, these experiments confirm that reduced TMPD is an effective substrate for this enzyme, based on the observation that the rate of O_2 consumption and the steady-state reduction level of the *a*-type cytochromes are correlated to TMPD concentration.

The results obtained from equilibrium and kinetics of CO binding to the reduced membranes provide additional indication that the protein coded by the *Sox*ABC genes is a canonical oxidase (even with respect to CO binding). Investigation of CO binding to ascorbate-reduced membranes shows indeed only one optical transition with affinity constant, spectral features and kinetic properties not very dissimilar from those characteristic of bovine oxidase (11). This is original evidence that the aa_3 terminal oxidase of *S. acidocaldarius* contains a binuclear heme-Cu center in an assembly structurally and functionally conserved during evolution, showing spectral and reactivity features very similar from archaebacteria to eukaryotes.

Direct indication that more than one chromophore contributes to the 586 nm band comes from the interpretation of the kinetic heterogeneity observed in the reaction with dithionite, where a faster component ($586^{(I)}$) is reduced synchronously with the 605 nm band (aa_3), while a second component ($586^{(II)}$) is reduced much more slowly (Fig. 1). The existence of two pools of *a*-type cytochromes both absorbing at 586 nm, but with different chemical properties, can be clearly demonstrated also in the reaction with ascorbate plus TMPD, where over and above reduction of the aa_3(605)-component, only a fraction ($586^{(I)}$) of the hemes *a* absorbing at 586 nm is reduced (Fig. 2) as compared to the total absorbance change observed upon addition of dithionite.

The observation that CO titration of the dithionite-reduced membranes displays two clearly distinct optical transitions (Fig. 5, *Panel A*), one being characterized by an unusually high affinity with spectral features atypical for an aa_3-type terminal oxidase, is also consistent with the hypothesis that this atypical binding site may indeed be one of the two pools of heme *a* absorbing at 586 nm ($586^{(II)}$), maybe becoming pentacoordinated upon reduction by dithionite.

Evidence that the second pool of *a*-586 ($586^{(II)}$) does not belong to the aa_3-type quinol oxidase comes from additional experiments carried out on the purified three-subunit oxidase,[2] which indicate that the enzyme: (i) is reduced by ascorbate plus TMPD to a level very similar to that obtained by dithionite, contrary to membranes; (ii) reacts with CO yielding optical, kinetic, and affinity properties not inconsistent with mitochondrial oxidase; and (iii) displays no heme *a* absorbing at 586 nm reacting with dithionite *very slowly*, as observed in native membranes. Therefore we believe that some of the heme *a*-586 component is not part of the *S. acidocaldarius* oxidase system as described here since it is not observed in the purified three-subunit enzyme. In the light of a very recent report by Lübben *et al.* (13), we conclude that the 586-nm component reacting with dithionite very slowly, as well as the additional CO binding site, may be part of an alternative terminal oxidase, although this remains to be seen.

Finally it may be useful to make a comment about the power of combining stopped-flow optical spectroscopy and SVD analysis for the investigation of *in situ* properties of membrane bound cytochromes in prokaryotes. Some of the difficulties involved may be appreciated from examination of the data, especially those in Fig. 4 (*Panel A*); total optical density changes of a few thousandths can be time-resolved and unequivocally fitted to two-exponential kinetic processes on top of an absolute optical density greater than 1. This type of approach is useful not only because it affords economy of material, but especially because it allows application of sophisticated kinetics to enzymes *in situ* with satisfactory spectral resolution and acceptable signal-to-noise ratio, and thereby it allows the comparison with the purified complexes.

In conclusion, we believe that the results described in this study are not inconsistent with the structural model presented by Lübben *et al.* (6), which accounts for the redox centers present in the enzymatic complex displaying oxidase activity. Some of the data suggest that, starting with the ascorbate plus TMPD-reduced enzyme, intramolecular electron transfer within this complex is very rapid. It may be speculated that the ascorbate plus TMPD-reduced enzyme is somehow functionally analogous to the fully reduced bovine oxidase (with four active redox centers), the role of Cu_A in the latter being replaced by one or two hemes *a* absorbing at 586 nm and bound to the subunit encoded by the *SoxC* gene. Moreover results provide evidence for an additional cytochrome absorbing at 586 nm ($586^{(II)}$) with kinetic properties quite distinct from those of the aa_3-type quinol oxidase.

Acknowledgment—We express our appreciation to Prof. P. Sarti for useful discussions.

REFERENCES

1. Lübben, M., and Schäfer, G. (1989) *J. Bacteriol.* **171,** 6106–6116
2. De Rosa, M., De Rosa, S., Gambacorta, A., Minale, L., Thomson, R. H., and Worthington, R. D. (1977) *J. Chem. Soc. Perkin Trans. I* **1,** 653–657
3. Wakao, H., Wakagi, T., and Oshima, T. (1987) *J. Biochem. (Tokyo)* **102,** 255–262
4. Moll, R., and Schäfer, G. (1991) *Eur. J. Biochem.* **201,** 593–600
5. Anemüller, S., and Schäfer, G. (1990) *Eur. J. Biochem.* **191,** 297–305
6. Lübben, M., Kolmerer, B., and Saraste, M. (1992) *EMBO J.* **11,** 805–812
7. Becker, M., and Schäfer, G. (1991) *FEBS Lett.* **291,** 331–335
8. Anemüller, S., Schmidt, C. L., Schäfer, G., and Teixeira, M. (1993) *FEBS Lett.* **318,** 61–64
9. Yonetani, T. (1961) *J. Biol. Chem.* **236,** 1680–1688
10. Henry, E. R., and Hofrichter, J. (1992) *Methods Enzymol.* **210,** 129–192
11. Gibson, Q., and Greenwood, C. (1963) *Biochem. J.* **86,** 541–555
12. Anemüller, S., Bill, E., Schäfer, G., Trautwein, A. X., and Teixeira, M. (1992) *Eur. J. Biochem.* **210,** 133–138
13. Lübben, M., Arnaud, S., Castresana, J., Warne, A., Albracht, S. P. J., and Saraste, M. (1994) *Eur. J. Biochem.* **224,** 151–159