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The binding of cyanide to cytochrome *d* in intact cells, spheroplasts, membrane fragments and solubilized enzyme from *Salmonella typhimurium*

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Abstract

This investigation focused on the kinetics of cyanide binding to oxidized and reduced cytochrome *d* in *Salmonella typhimurium* intact cells, spheroplasts, membrane fragments and solubilized enzyme, and on the effect of pH on this binding. Cyanide bound to the oxidized form of cytochrome *d* under all experimental conditions, inducing a trough at 649 nm in the oxidized-cyanide-minus-oxidized difference absorption spectra. V_{max} of cyanide binding to oxidized cytochrome *d* at pH 7.0 was 14.0 ± 2.0 pmol/min/mg protein (prot.) in intact cells, 37.0 ± 3.5 pmol/min/mg prot. in spheroplasts, 125.0 ± 6.0 pmol/min/mg prot. in membrane fragments, and 538.0 ± 8.5 pmol/min/mg prot. in solubilized cytochrome *d*. The pseudo-first order rate constants were 0.004 s^{-1} for intact cells, 0.005 s^{-1} for spheroplasts, 0.007 s^{-1} for membrane fragments and 0.025 s^{-1} for the solubilized enzyme. The V_{max} value was highest at pH 7.0 for intact cells and solubilized cytochrome *d* and at pH 8.0 for both spheroplasts and membrane fragments. The K_s of binding at pH 7.0 was around 4 mM in intact cells, spheroplasts and membrane fragments, but was 10.5 mM in solubilized cytochrome *d*. This difference between the K_s values suggested a change in conformation, upon solubilization, leading to a decrease in the affinity of cyanide for the solubilized enzyme. The K_s value was nearly the same at all pH investigated (pH 5–10). Cyanide was found to also bind to the reduced form of cytochrome *d* in membrane fragments (K_s = 18 ± 3 mM, V_{max} = 377 ± 28 pmol/min/mg prot. at pH 7) and the solubilized enzyme (K_s = 18 ± 1.2 mM, V_{max} = 649 ± 45 pmol/min/mg prot. at pH 7) with a lower affinity of cyanide for the reduced cytochrome *d* than for the oxidized enzyme. Pseudo-first order rate constants were 0.025 s^{-1} and 0.042 s^{-1} respectively for membrane fragments and solubilized enzyme. The value of V_{max} for cyanide binding to the reduced cytochrome *d*, whether membrane-bound or solubilized, increased slightly with pH (for pH 6–10) while the K_s value dropped significantly with increasing pH. The pH dependence observed here might be interpretable as a possible role for conformational transition associated with energy transduction. Finally, this investigation pointed to the influence of the microenvironment of a protein within the cell on its reactivity.

Keywords: Cytochrome *d*; Kinetics; Ligand; Cyanide; pH; *Salmonella typhimurium*

1. Introduction

Salmonella typhimurium is a Gram negative and facultative anaerobic bacterium which belongs to the Enterobacteriacea genera. *Escherichia coli* also

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belongs to this group and is very similar to *S. typhimurium* in many features. Homologous genes of these bacteria have an average of 85% sequence identity [1].

Facultative anaerobic bacteria can extract energy from substrate both aerobically and anaerobically, but without oxygen and other electron acceptors, energy production depends upon fermentation [2] which is less efficient than the aerobic metabolism. In contrast to eukaryotic cells, bacteria have different kinds of cytochromes in their respiratory chains [3], including different terminal oxidases [4] which enable them to use the aerobic metabolism even in the presence of a limited amount of oxygen. The respiratory chain of *E. coli* has been extensively studied [5,6]. It includes two terminal oxidases, the cytochrome *bo* complex and the cytochrome *bd* complex, which are quinol oxidases [3]. Expression of the cytochrome *bo* complex, which functions as a coupling site in the respiratory chain [7], is elevated during aerobiosis [8,9]. Cytochrome *o* belongs to the family of oxidases which contain a heme/copper binuclear oxygen reducing center and which function as proton pump [10]. When the oxygen concentration drops in the culture medium, the cytochrome *bo* complex is replaced by the cytochrome *bd* complex which is then predominantly synthesized in the cells [9,11]. Thus the major terminal oxidase found in cells in the stationary phase of culture is the cytochrome *bd* complex [12]. This complex has been characterized with a higher affinity for oxygen than the cytochrome *bo* complex [4,13–17]. Furthermore, it is generally recognized that the cytochrome *bd* complex transfers electrons to oxygen with formation of a proton gradient but without invoking a proton pump [13–16]. Extensive spectrophotometric studies, MCD, ESR and potentiometric titrations have shown that the *bd* complex exhibits three redox centers, namely the low spin heme b_{558} and the two high spin hemes b_{595} and *d* [18–20]. Cytochrome *d* shows an α absorption band at 630 nm but little if any contribution in the Soret region [4,15,16]. In contrast, cytochromes b_{558} and b_{595} show both α and Soret absorption bands [15,16]. Interaction of various ligands with *bd*-type cytochromes has been investigated ([20] and references therein). Cyanide is a known respiration inhibitor and its interaction with oxidases of various respiratory chains has been reviewed in the past [21], including its interaction with mammalian cytochrome *c* oxidase ([22] and references therein) as well as with cytochrome *d* of *E. coli* [23] and *Azotobacter vinelandii* [24,25]. Cyanide was found to bind to the oxidized state of cytochrome *d* (Fe^{3+}) that eventually forms from the oxygenated enzyme [4,15,16,26,27], but it was not found to bind to the reduced form of cytochrome *d* [4,11,24]. However, more recent studies on isolated cytochrome *bd* oxidase from *E. coli* mention binding to the fully reduced enzyme upon addition of a large excess of cyanide (approx. 35 mM) [27]. Of course the cyanide anion is known to bind more strongly to ferric than to ferrous heme and this has been shown in other hemoproteins such as horseradish peroxidase [28]. Virtually most studies, including recent ones [16,26,27], have payed very little attention to other bacterial species. Moreover, despite numerous studies on the interaction between cyanide and cytochrome *d*, detailed studies on the effect of various experimental conditions such as variable pH, intact cells versus membrane fragments, or further investigations on the interaction with reduced cytochrome *d* are still lacking. In this research we investigate the effect of cyanide on oxidized and reduced cytochrome *d* in *S. typhimurium* intact cells, spheroplasts, membrane fragments and Triton X-100 solubilized cytochrome *d*, and the effect of pH on this binding. A preliminary report of this research has been published in [29,30] and presented in a FEBS meeting [31].

2. Materials and methods

2.1. Bacterial strain

S. typhimurium strain 3507 from Dr. Roseman's laboratory at Johns Hopkins University was provided to us by Dr. N.O. Keyhani.

Cells were grown in enriched medium (7 g peptone, 5 g yeast extract, 3 g NaCl per liter, pH 7.0) for 48 h at 37°C in a rotary shaker at 150 rpm.

2.2. Cell harvesting

Cells were harvested by centrifugation (5500×*g*); the cell pellet was washed twice in phosphate buffer 0.1 M, pH 7.0 (centrifugation at 8500×*g*).

2.3. Spheroplast preparation

Spheroplasts were prepared according to [32]. Spheroplast formation was monitored by following the decrease in optical density of the cell suspensions.

2.4. Membrane fragment preparation

Spheroplasts were suspended in Tris buffer 0.05 M, pH 9.0. The suspension was homogenized in a Waring blender, then centrifuged at $10\,000 \times g$ for 40 min. The yellow cloudy supernatant consisting of membrane fragments and soluble proteins was centrifuged at $40\,000 \times g$ for 40 min. The pellet consisting of membrane fragments was washed once with phosphate buffer 0.1 M, pH 7.0 and recentrifuged at $40\,000 \times g$ for 40 min.

2.5. Preparation of solubilized cytochrome d

To prepare soluble cytochrome *d*, membrane fragments were treated with 1% Triton X-100 in 0.1 M phosphate buffer, pH 7.0, at room temperature (approx. 25°C) for 45 min, then centrifuged at $50\,000 \times g$ for 40 min. The pale, yellow supernatant containing cytochrome *d* was used for experimentation and the pellet discarded.

2.6. Absorption spectra

Room temperature reduced-minus-oxidized difference spectra of cell suspensions were recorded with an Aminco DW2 spectrophotometer according to [33,34] with some modifications as described below.

2.7. Kinetics of cyanide binding to cytochrome d

To monitor cyanide binding to oxidized cytochrome *d*, intact cells and spheroplasts were oxidized with H_2O_2 (8 mM) and placed in both the sample and the reference cuvettes. In preliminary experiments, both cuvettes were air oxidized. However, under those conditions, anaerobiosis occurred within about 5 min. This interfered with accurate assays of the cytochrome *d*-CN complex, leading to results difficult to reproduce and unreliable. Therefore we decided to use H_2O_2 to keep the system in the oxidized state throughout our kinetic experiments. The presence of catalase in the cells and spheroplasts caused decomposition of H_2O_2, thus enough O_2 was present to keep the system in the oxidized state. Although under these conditions cytochrome *d* was likely to be essentially oxygenated at first, it has been well established that cyanide binds to the oxidized state (Fe^{3+}) that eventually forms [4,15,16,26,27]. Therefore we referred in this work to the oxidized form of cytochrome *d* only.

No catalase was detectable in membrane fragments or solubilized cytochrome *d* preparations. Thus to ensure that cytochrome *d* remained in the oxidized state in these preparations, each of the three following experimental conditions were used: (1) air oxidation; (2) oxidation with H_2O_2 (8 mM) and added catalase (125 nM); (3) oxidation with a few grains of ammonium persulfate.

Neutralized KCN (up to 30 mM) was added to the sample cuvette and oxidized+KCN-minus-oxidized difference absorption spectra were recorded at various time intervals. The binding of cyanide to cytochrome *d* was measured as the depth of the trough at 649 nm, using an extinction coefficient of 11 mM^{-1} cm^{-1} at 690–649 nm which was calculated according to [35]. For studies with reduced samples, membrane fragments or solubilized cytochrome *d* were reduced with a few grains dithionite before addition of neutralized KCN (up to 80 mM); references were identical to the samples, except for KCN. The binding of cyanide to cytochrome *d* was measured as the depth of the trough at 635 nm, using an extinction coefficient of 16 mM^{-1} cm^{-1} at 690–635 nm which was calculated according to [35].

Unless otherwise specified, all assays were conducted in 0.1 M phosphate buffer at pH 7.0.

3. Results

The reduced-minus-oxidized difference absorption spectrum of *S. typhimurium* intact cells grown to stationary phase is shown in Fig. 1A. The spectrum exhibited an absorption peak at 630 nm and a trough at 649 nm due to cytochrome *d*, a small absorption peak at 595 nm due to cytochrome b_{595}, a peak at 560 nm due to cytochrome b_{560} and a peak at 435 nm (Soret region) due to the γ band of *b*-type cytochromes [15,16]. The effect of cyanide on cytochrome

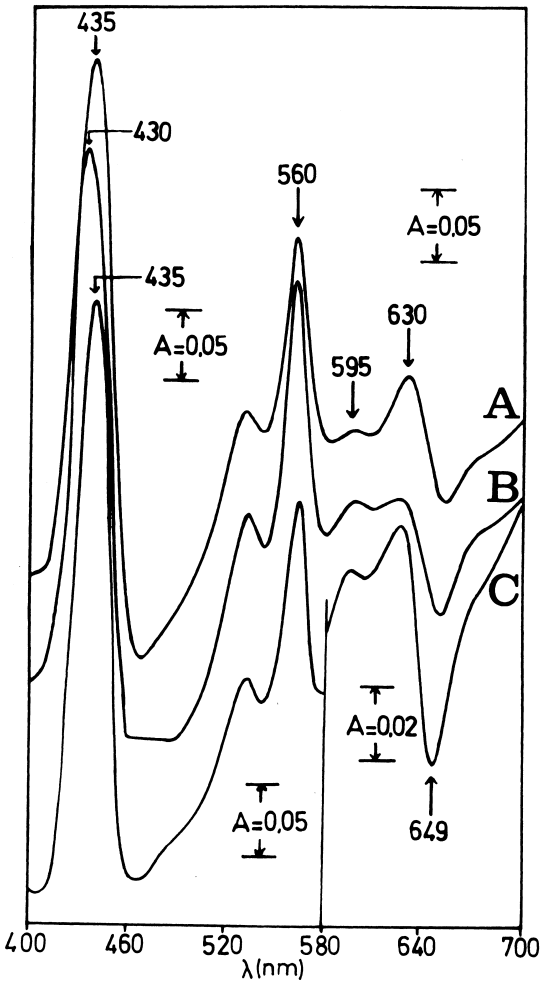

Fig. 1. Difference absorption spectra of *S. typhimurium* intact cell suspensions. (A) Dithionite reduced-minus-H_2O_2 oxidized difference spectrum. The sample was reduced with dithionite and the reference was oxidized by addition of 8 mM H_2O_2. (B) Effect of KCN on oxidized cytochrome *d* in intact cells: H_2O_2 oxidized sample-KCN-minus-H_2O_2 oxidized sample difference spectrum. Both sample and reference were kept in the oxidized state by the addition of 8 mM H_2O_2; then KCN (1 mM) was added to the sample. The complex of oxidized cytochrome *d*-CN showed a trough at 649 nm. (C) H_2O_2 oxidized sample-KCN+dithionite-minus-H_2O_2 oxidized sample difference spectrum. Upon addition of dithionite to the sample, some reduction of cytochrome *d* was detected as the absorption band at 630 nm appeared; the Soret band shifted back to 435 nm. (A,B) 26 mg prot./ml; (C) 32 mg prot./ml.

d in intact cells is shown in Fig. 1B. Both sample and reference were kept in the oxidized state by the addition of 8 mM H_2O_2, and KCN (1 mM) was then added to the sample; the complex formed between oxidized cytochrome *d* and cyanide showed a trough at 649 nm without the peak at 630 nm. Furthermore, a shift in the Soret band was observed from 435 to 430 nm. Upon addition of dithionite to the sample, some reduction of cytochrome *d* was detected as the absorption band at 630 nm reappeared (Fig. 1C); simultaneously, the Soret band shifted back from 430 nm to 435 nm. However, in agreement with earlier reports that cyanide inhibits reduction of heme *d* [15,24,26,36], the results shown in Fig. 1C were not consistently observed.

Cyanide was found to bind to the oxidized form of cytochrome *d* in spheroplasts, membrane fragments and solubilized enzyme as well as in intact cells. Furthermore, cyanide also bound to the reduced form of cytochrome *d* in membrane fragments and solubilized enzyme. Fig. 2 shows the effect of cyanide on solubilized cytochrome *d* obtained from Triton-treated membranes as described in Section 2. Addition of 20 mM KCN to the oxidized form of solubilized cytochrome *d* resulted in the formation of a trough at 648 nm which progressively increased with time, as shown in Fig. 2A, when ammonium persulfate oxidized-KCN-minus-ammonium persulfate oxidized difference absorption spectra were recorded. In contrast, addition of 20 mM KCN to dithionite-reduced solubilized cytochrome *d* resulted in the progressive formation of a trough at 635 nm and two minor absorption bands at 599 nm and 678 nm (Fig. 2B) when dithionite reduced-KCN-minus-dithionite reduced difference absorption spectra were recorded. Thus a shift of 13 nm (from 648 nm to 635 nm) was observed between the trough due to the oxidized cytochrome *d*-CN complex and the trough due to the reduced cytochrome *d*-CN complex.

Fig. 3 shows the effect of increasing amounts of KCN on the formation of the oxidized cytochrome *d*-CN complex in intact cells, spheroplasts, membrane-bound and solubilized cytochrome *d* (Fig. 3A) and on the formation of dithionite-reduced cytochrome *d*-CN complex in membrane-bound and solubilized cytochrome *d* (Fig. 3B). Binding of cyanide to oxidized cytochrome *d* in intact cells, spheroplasts and membrane fragments was roughly parallel, reaching a plateau at 84 pmole complex per mg protein in intact cells, 110 pmole complex per mg protein in spheroplasts, and 148 pmole complex per mg protein in membrane fragments, for 15 mM

KCN (Fig. 3A). The amount of cytochrome *d*-CN complex formed with the oxidized form of solubilized cytochrome *d* also reached a plateau around 15 mM KCN; however, the amount of complex formed, 370 pmole per mg protein, was almost 2.5 times higher than the amount of complex formed with membrane-bound cytochrome *d* (Fig. 3A). For each condition, the maximum amount of complex was formed after 15 min incubation at room temperature. In contrast, when cytochrome *d*, either membrane-bound or solubilized, was reduced, the amount

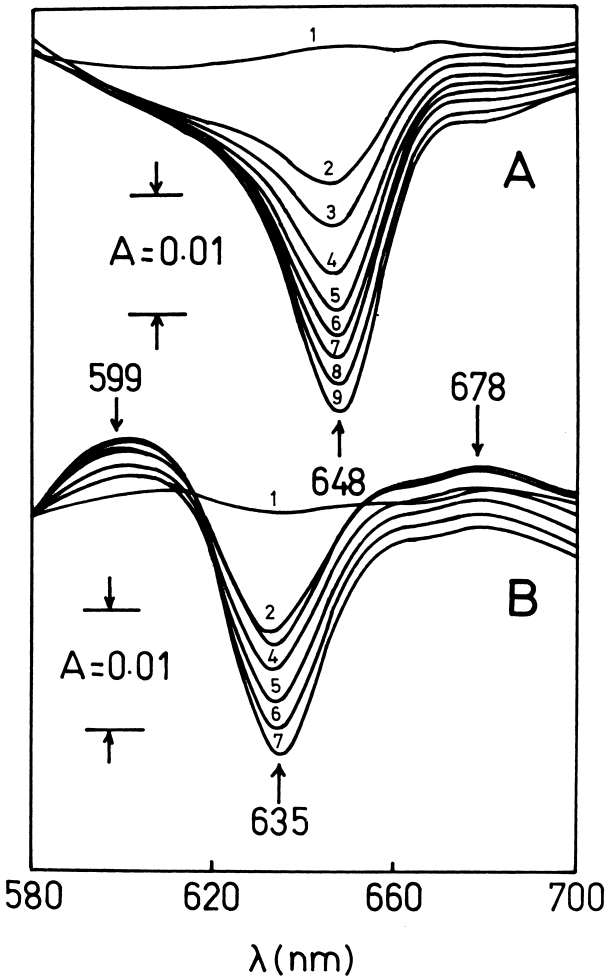

Fig. 2. Formation of cytochrome *d*-CN complex upon addition of KCN (20 mM) to solubilized cytochrome *d* in 0.1 M phosphate buffer, pH 7.0. (A) Ammonium persulfate oxidized sample-KCN-minus-air oxidized sample (11.5 mg prot./ml); (B) dithionite-reduced sample KCN-minus-dithionite-reduced sample (5.75 mg prot./ml). Difference spectra were recorded at 1 min intervals. The figure shows a progressively deepening trough at 648 nm for the oxidized form (A) and at 635 nm for the reduced form (B) of cytochrome *d*.

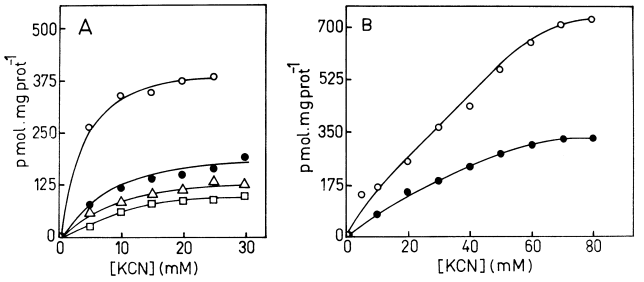

Fig. 3. Effect of increasing concentrations of KCN on the formation of cytochrome *d*-CN complex in intact cells (□), spheroplasts (△), membrane fragments (●) and solubilized enzyme (○). (A) Oxidized samples; incubation for 15 min at room temperature with KCN in 0.1 M phosphate buffer, pH 7.0; (B) dithionite-reduced samples; incubation for 5 min at room temperature with KCN in 0.1 M phosphate buffer, pH 7.0. (A) Note that the amount of complex formed between the oxidized cytochrome *d* and cyanide was increasing as a function of KCN concentration until 15 mM KCN; thereafter the amount of complex formed remained roughly constant. The amount of complex found in the solubilized enzyme preparation was roughly 2.5 times higher than in the other preparations. (B) The amount of complex formed between the dithionite-reduced cytochrome *d* and cyanide increased as a function of KCN concentration until 60 mM KCN; thereafter the amount of complex formed remained roughly constant. It was twice the amount found for the oxidized samples and the amount of complex found in the solubilized enzyme preparation was still roughly 2.5 times higher than in the membrane fragments preparation. (A,B) Each point is the average of three separate experiments.

of complex formed with cyanide reached a plateau only for 80 mM KCN (Fig. 3B). The amount of cytochrome *d*-CN complex formed per mg protein was still about 2.5 times higher for solubilized cytochrome *d* (700 pmole complex) than for membrane-bound cytochrome *d* (300 pmole complex) (Fig. 3B). Furthermore, the amount of complex formed with the reduced cytochrome *d* was twice the amount of complex formed with the oxidized cytochrome *d* (Fig. 3B compared to Fig. 3A). For both membrane-bound and solubilized cytochrome *d*, the maximum amount of complex was formed after 5 min incubation, at room temperature, with any of the tested KCN concentrations (5–80 mM). Thus, unlike the observations made in intact cells and spheroplasts in which cyanide bound only to the oxidized form of cytochrome *d* ([4,11]; this paper), both the oxidized and the reduced form of cytochrome *d* would form a complex with cyanide in membrane fragments and in the soluble fraction. Interestingly,

more complex would form in a shorter amount of time with the reduced enzyme than with the oxidized enzyme, whether cytochrome *d* was solubilized or membrane-bound. Fig. 4 shows the second order rate constants for the reaction with the oxidized and the reduced enzyme in intact cells, spheroplasts, membrane fragments and solubilized enzyme at various KCN concentrations. For the oxidized preparations, the rate constant was lowest in intact cells and spheroplasts and it decreased slightly with increasing KCN concentrations. At 5 mM KCN, the second order rate constant was 0.3 M^{-1} s^{-1} in intact cells and 0.4 M^{-1} s^{-1} in spheroplasts, while at 25 mM KCN, it was 0.17 M^{-1} s^{-1} in intact cells and 0.19 M^{-1} s^{-1} in spheroplasts. It was more dependent on cyanide concentration in membrane fragments and, especially, in the solubilized enzyme although the dependence would lessen with increasing KCN concentrations. At 5 mM KCN, the second order rate constant was 0.74 M^{-1} s^{-1} in membrane fragments and 3.4 M^{-1} s^{-1} for the solubilized enzyme, while at 25 mM KCN, it was 0.28 M^{-1} s^{-1} in membrane fragments and 1.00 M^{-1} s^{-1} in the solubilized enzyme (Fig. 4A). For KCN concentrations above 15 mM, the pseudo-first order rate constant was lowest in intact cells (0.004 s^{-1}) and spheroplasts (0.005 s^{-1}); however, compared to intact cells, its value almost doubled in membrane fragments (0.007 s^{-1}), and was 6 times higher for the solubilized enzyme (0.025 s^{-1}). The second order rate constant for the reaction between the reduced enzyme and cyanide was higher in the solubilized enzyme than in the membrane fragments (Fig. 4B). In both cases,

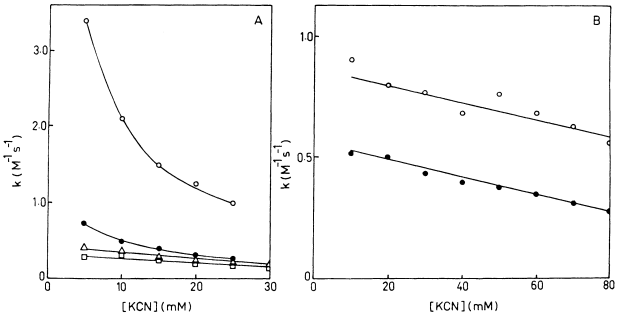

Fig. 4. Effect of increasing concentrations of KCN on the second order rate constant for the formation of cytochrome *d*-CN complex in intact cells (□), spheroplasts (△), membrane fragments (●) and solubilized enzyme (○). (A) Oxidized samples; (B) reduced samples.

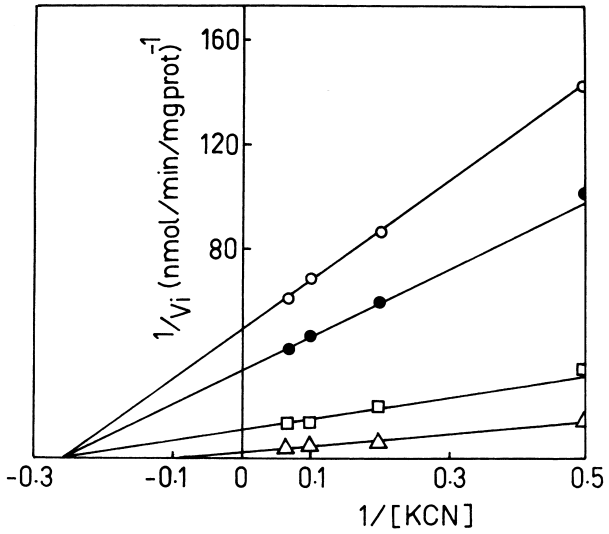

Fig. 5. Lineweaver-Burk plot for the formation of oxidized cytochrome *d*-CN complex in intact cells (○), spheroplasts (●), membrane fragments (□), and solubilized cytochrome *d* (△). Note that K_s was similar (4 mM) for intact cells, spheroplasts and membrane fragments, but was higher (10.5 mM) for solubilized cytochrome *d*. The highest value for V_{max} was found for the solubilized enzyme (435 ± 7 mM) and the lowest value was found for intact cells (11.25 ± 1.7 pmol/min/mg prot.). [KCN] was mM. Oxidation of intact cells and spheroplasts was done in the presence of 8 mM H_2O_2; oxidation of membrane-bound and solubilized cytochrome *d* was done with a few grains of ammonium persulfate.

it decreased slightly with increasing KCN concentrations. At 10 mM KCN, it was 0.51 M^{-1} s^{-1} in membrane fragments and 1.1 M^{-1} s^{-1} in the solubilized enzyme; at 80 mM KCN, it was 0.28 M^{-1} s^{-1} in membrane fragments and 0.56 M^{-1} s^{-1} in the solubilized enzyme. For KCN concentrations above 60 mM the pseudo-first order rate constant was 0.02 s^{-1} in membrane fragments (3 times the value obtained with the oxidized membrane-bound enzyme) and 0.044 s^{-1} in the solubilized enzyme (2 times the value obtained with the oxidized enzyme).

Lineweaver-Burk plots (Fig. 5) for the reaction between oxidized cytochrome *d* and cyanide at pH 7 in intact cells, spheroplasts, membrane fragments and the solubilized cytochrome *d* showed that K_s was 4 ± 0.50 mM in the first three conditions but was equal to 10.4 ± 0.35 mM in the solubilized cytochrome *d*. V_{max} was higher in membrane fragments (125.0 ± 6.0 pmol/min/mg protein) than in sphero-

Table 1
Kinetic parameters of cyanide binding to membrane-bound and solubilized cytochrome *d* under different oxidation conditions at pH 7.0

Oxidation method	K_s (mM)	V_{max} (pmol/min/mg prot)	k (s^{-1})
Membrane-bound enzyme			
Air oxidized	4.8 ± 0.6	136 ± 5	0.008
Oxidized with H_2O_2 and catalase	4.4 ± 0.4	98 ± 6	0.006
Oxidized with persulfate	4.0 ± 0.5	125 ± 6	0.007
Solubilized enzyme			
Air oxidized	10.2 ± 0.4	385 ± 10	0.032
Oxidized with H_2O_2 and catalase	11.2 ± 0.6	318 ± 18	0.026
Oxidized with persulfate	10.4 ± 0.35	538 ± 8.5	0.025

All results are averages of three separate experiments.

plasts (37.0 ± 3.5 pmol/min/mg protein), and it was lowest in intact cells (14.0 ± 2.0 pmol/min/mg protein); V_{max} was highest in solubilized cytochrome *d* (538.0 ± 8.5 pmol/min/mg protein).

Results obtained with membrane fragments and solubilized enzyme oxidized with ammonium persulfate were similar to those obtained when the preparations were either air oxidized or oxidized in the presence of H_2O_2 and catalase as shown in Table 1. K_s was found to be around 4 mM with the membrane-bound enzyme, regardless of the oxidation method used (4.8 ± 0.6 mM when preparations were air oxidized, 4.4 ± 0.4 mM when the preparations were oxidized in the presence of H_2O_2 and catalase, and 4.0 ± 0.5 mM when preparations were oxidized with ammonium persulfate); it was found to be around 10.5 mM with the solubilized enzyme, regardless of the oxidation method used (10.2 ± 0.4 mM when preparations were air oxidized, 11.2 ± 0.6 mM when preparations were oxidized in the presence of H_2O_2 and catalase, and 10.4 ± 0.35 mM when preparations were oxidized with ammonium persulfate). The pseudo-first order rate constant was around 0.007 s^{-1} with the membrane-bound enzyme under all oxidation conditions (0.008 s^{-1} when air oxidation was used, 0.006 s^{-1} when oxidation in the presence of H_2O_2 and catalase was used, and 0.007 s^{-1} when oxidation with ammonium persulfate was used); it was around 0.028 s^{-1} with the solubilized enzyme (0.032 s^{-1} when the enzyme was air oxidized, 0.026 s^{-1} when the enzyme was oxidized in the presence of H_2O_2 and catalase, and 0.025 s^{-1} when the enzyme was oxidized with ammonium persulfate). V_{max} varied by about 20%, depending on the oxidation method, for the membrane-bound enzyme (136 ± 5 pmol/min/mg protein for air oxidation, 98 ± 6 pmol/min/mg protein for oxidation in the presence of H_2O_2 and catalase, and 125 ± 6 pmol/min/mg

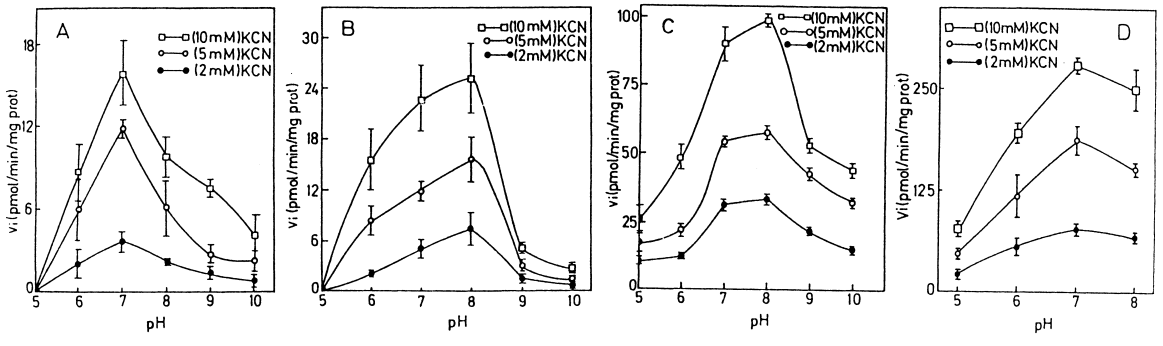

Fig. 6. pH profile of KCN binding to oxidized cytochrome *d*. (A) Intact cells; (B) spheroplasts; (C) membrane fragments; (D) solubilized enzyme. Optimum pH for intact cells and solubilized cytochrome *d* was 7. In contrast, optimum pH for spheroplasts and membrane fragments was 8. Note that for solubilized enzyme pH over 8 led to the denaturation of cytochrome *d* and the absence of any binding to cytochrome *d*.

protein for oxidation with ammonium persulfate). For the solubilized enzyme, V_{max} was highest for the preparations oxidized with ammonium persulfate (538 ± 8.5 pmol/min/mg protein); it dropped by 30% in air oxidized preparations (385 ± 10 pmol/min/mg protein) and by 40% in the preparations oxidized in the presence of H_2O_2 and catalase (318 ± 18 pmol/min/mg protein (prot.)).

The effect of pH (Fig. 6A–D) on the rate of formation of the cytochrome *d*-CN complex when cytochrome *d* was in the oxidized form showed that optimum rate of binding in both spheroplasts (B) and membrane fragments (C) was observed at pH 8.0. With intact cells (A) and solubilized cytochrome *d* (D) the optimum rate of binding was found at pH 7.0. Increasing the pH to 10.0 or decreasing it to 5.0 reduced drastically the rate of binding in all cases. For solubilized enzyme, cyanide did not bind to cytochrome *d* at pH above 8.0 (Fig. 5D).

Fig. 7 shows the pH dependence of K_s (Fig. 7A), V_{max} (Fig. 7B) and the pseudo-first order rate constant (Fig. 7C) for the formation of oxidized cytochrome *d*-CN complex and reduced cytochrome *d*-CN complex in membrane-bound and in solubilized preparations. With the oxidized enzyme, the results in Fig. 7 show that for membrane-bound cytochrome *d*-CN, K_s remained constant around 4 mM. In contrast, V_{max} was lowest at pH 5 (34 pmol/min/mg prot.), progressively rose to 141 pmol/min/mg prot. at pH 8 and decreased thereafter to reach 59 pmol/min/mg prot. at pH 10. The pseudo-first order rate constant was also pH-dependent with a maximum value of 0.007 s^{-1} at pH 8.0. K_s for solubilized oxidized cytochrome *d*-CN complex was around 12 mM at pH 5 and 6 and around 10.5 mM at pH 7 and 8; at pH 9 and 10, complex formation did not occur, presumably due to the denaturation of cytochrome *d* and its inability to bind cyanide. V_{max} for solubilized enzyme rose progressively from 171 pmol/min/mg prot. at pH 5 to 504 pmol/min/mg prot. at pH 8, peaking at 538 pmol/min/mg prot. at pH 7; it remained zero at pH 9 and 10. The pseudo-first order rate constant was pH-dependent at lower pH (pH 5.0 and 6.0) and reached a peak value of 0.03 s^{-1} at pH 7.0.

With the reduced enzyme, the pH dependence of K_s, V_{max} and the pseudo-first order rate constant was studied from pH 6 to pH 10. At pH 5 no stably reduced cytochrome *d* could be formed. The results in Fig. 7 show that for membrane-bound cytochrome *d*-CN, K_s was highest (21 mM) at pH 6; it then decreased progressively, reaching 16 mM at pH 10. V_{max} was lowest at pH 6 (344 pmol/min/mg prot.), then progressively rose to 412 pmol/min/mg prot. at pH 8, 9 and 10. The pseudo-first order rate constant was pH-independent, remaining at the value of 0.025 s^{-1} which was 3 times the maximum value found with the oxidized enzyme. K_s for soluble reduced cytochrome *d*-CN decreased appreciably with increasing pH. From 28 mM at pH 6, K_s decreased progressively to 11 mM at pH 8; at pH 9 and 10, no complex was formed. V_{max} for the formation of soluble reduced cytochrome *d*-CN complex increased progressively from 553 pmol/min/mg prot. at pH 6 to 680 pmol/min/mg prot. at pH 8. The pseudo-first order rate constant was pH-dependent, increasing from 0.036 s^{-1} at pH 6.0 to 0.043 s^{-1} at pH 8.0, which was 1.5 times the value found for the oxidized enzyme (0.03 s^{-1}).

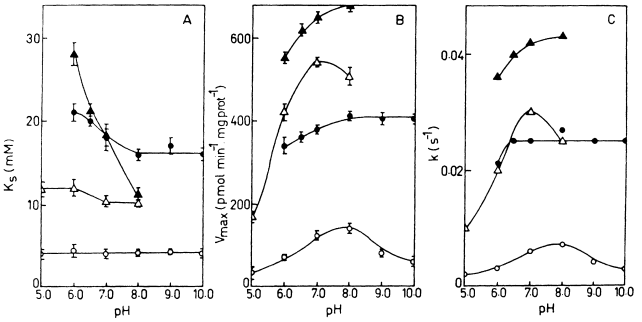

Fig. 7. pH dependence of K_s (A), V_{max} (B) and pseudo-first order rate constant (C) for the formation of cytochrome *d*-CN complex with membrane-bound and solubilized enzyme. Samples were oxidized with a few grains of ammonium persulfate or reduced with a few grains of dithionite. ○, oxidized membrane-bound enzyme; △, oxidized solubilized enzyme; ●, reduced membrane-bound enzyme; ▲, reduced solubilized enzyme.

4. Discussion

Like *E. coli* [4], *S. typhimurium* synthesized cytochrome *d* in the stationary phase of growth. In *S. typhimurium* intact cells, cyanide bound to cytochrome *d* in the oxidized state, forming a complex identifiable by a trough at 649 nm in oxidized-KCN-

minus-oxidized difference absorption spectra. This characteristic trough at 649 nm had been identified for the cytochrome *d*-CN complex in *E. coli* as well [16]. Cyanide also bound to oxidized cytochrome *d* in spheroplasts, membrane fragments and the solubilized enzyme from *S. typhimurium*. The complex thus formed could not be easily reduced by dithionite as shown in Fig. 1C (intact cells) and as was shown by others in *E. coli* [26], *A. vinelandii* [24,36] and even in other hemoproteins such as horseradish peroxidase [28]. Cyanide also bound to cytochrome *d* in the reduced state in membrane fragments and in the solubilized enzyme preparations from *S. typhimurium*. However, the trough characteristic of the reduced cytochrome *d*-CN complex formed with the solubilized enzyme showed a 13 nm blue shift from 648 nm (oxidized enzyme) to 635 nm (reduced enzyme) (Fig. 2).

The kinetic parameters collected in this work indicated a definite difference of behavior towards cyanide between the oxidized and the reduced cytochrome *d*. Interestingly, while the affinity for cyanide was higher in the oxidized enzyme than in the reduced enzyme, the rate of reaction was lower with the oxidized enzyme. pH also affected differently the reaction between cyanide and the oxidized enzyme and the reaction between cyanide and the reduced enzyme. For the oxidized enzyme, the affinity for cyanide was unaffected by pH while the rate of reaction was pH-dependent. On the other hand, for the reduced enzyme, the affinity for cyanide diminished as the pH decreased. It was similarly reported that the affinity of cyanide for ferric horseradish peroxidase was independent of pH but that the affinity of cyanide for ferrous peroxidase decreased with decreasing pH [28]. However, in horseradish peroxidase the K_d at pH 6.5 was reported to be 20 times larger than the K_d at pH 8.5, while in our case the K_s at pH 6.0 was just 3 times larger than the K_s at pH 8.0 for the solubilized enzyme; for the membrane-bound enzyme, the K_s at pH 6.0 was only 1.3 times larger than the K_s at pH 8.0.

The affinity of the oxidized cytochrome *d* for cyanide, as measured by the K_s at pH 7.0, was the same whether the enzyme was in intact cells, spheroplasts or membrane fragments but diminished 2.5 times when the enzyme was solubilized (Fig. 5). On the other hand, the rate of formation of the complex as measured by the V_{max} at pH 7.0 was highest in the solubilized enzyme (40 times higher than in intact cells, 15 times higher than in spheroplasts, 4 times higher than in membrane fragments) (Fig. 5), possibly reflecting a change in conformation upon solubilization. Pseudo-first order rate constants for the reaction in intact cells and spheroplasts were of the same order of magnitude as that found for the slow component of the reaction observed with the membrane-bound cytochrome *bd* in *E. coli* [16]. Unlike experiments such as CO flash photolysis of cytochrome *bd* [37], our experimental conditions and instrumentation did not allow us to identify kinetically distinct phases of KCN attachment to cytochrome *d*.

When the oxidized cytochrome *d* was incubated with increasing concentrations of KCN, the amount of complex formed increased until a plateau was reached for 15 mM KCN whether the enzyme was in intact cells, spheroplasts, membrane fragments or solubilized. The amount of complex formed per mg protein was about 2.5 times higher for solubilized cytochrome *d* than for membrane-bound cytochrome *d* (Fig. 3). This indicated that cyanide binding was facilitated in the solubilized enzyme when compared to membrane fragments, spheroplasts or intact cells, in spite of the lower affinity of the solubilized enzyme. pH (between 5.0 and 10.0) did not affect the affinity of the oxidized enzyme for cyanide in membrane fragments, and for the solubilized enzyme, the affinity was only slightly diminished at pH 5.0 and 6.0 (Fig. 7). V_{max} was pH-dependent, being highest at pH 7.0 and 8.0, whether the enzyme was membrane-bound or solubilized (Fig. 7).

In contrast, when cytochrome *d* was reduced, its affinity for cyanide, as measured by the K_s at pH 7.0, was the same for the membrane-bound and the solubilized enzyme. The rate of formation of the complex, as measured by the V_{max} at pH 7.0, was 1.7 times higher in the solubilized cytochrome *d* than in the membrane-bound enzyme. When the reduced cytochrome *d*, membrane-bound or solubilized, was incubated with increasing concentrations of KCN, a plateau was reached at 80 mM KCN (Fig. 3). The amount of complex per mg protein was still about 2.5 times higher for the solubilized cytochrome *d* than for the membrane-bound enzyme. Furthermore, the amount of complex formed with

the reduced enzyme was 2 times the amount formed with the oxidized enzyme (Fig. 3). This observation showed that the reduced cytochrome *d* had a much greater ability to bind cyanide than the oxidized enzyme in spite of lower affinity. The affinity of the reduced cytochrome *d* for cyanide was affected by pH. When the pH varied from 6.0 to 10.0, the affinity increased 1.5 times for the membrane-bound enzyme; when the pH varied from 6.0 to 8.0, the affinity increased 2.5 times for the solubilized enzyme (Fig. 7). V_{max} for the formation of the reduced cytochrome *d*-CN complex was less dependent on the pH for the membrane-bound enzyme than for the solubilized enzyme; it increased by 18% for the membrane-bound enzyme and by 25% for the solubilized enzyme when the pH increased from 6.0 to 8.0 (Fig. 7).

The optimum pH for the binding of cyanide to cytochrome *d* in *S. typhimurium* was lower in intact cells and solubilized enzyme (pH 7.0) than in both spheroplasts and membrane fragments (pH 8.0) (Fig. 6). Removal of the outer membrane which can affect the passage of solutes and ions, could be the main factor responsible for this difference between intact cells and both spheroplasts and membrane fragments.

Our results showed that the formation of the cytochrome *d*-CN complex was greatly influenced by changes in the experimental conditions ([29,30]; this paper). Differences in the K_s and V_{max} for the formation of the membrane-bound CN-inhibited cytochrome *d* and for the formation of the solubilized CN-inhibited cytochrome *d* were consistent with the observation of Merle and Kadenbach [38] who showed that for cytochrome *c* oxidase the K_m and V_{max} values differed in the membrane-bound and the deoxycholate solubilized enzyme. Thus, like cytochrome *c* oxidase reported in [38], the cytochrome *d* oxidase reported here behaved kinetically differently in the membrane-bound state and in the solubilized state. V_{max} for the formation of the complex between cytochrome *d* (oxidized or reduced) and cyanide was sensitive to changes in the pH, especially in the solubilized enzyme, indicating that the conformation of both ferri- and ferro-cytochrome *d* in the cyanide inhibited enzyme was sensitive to proton binding, presumably in the active site of the enzyme. The presence of an ionizable group near the active site of the cytochrome *bd* complex has been reported [19] and it has been suggested that a protonation site close to heme b_{595} may be involved in providing substrate protons [15]. Furthermore, both membrane-bound and solubilized cytochrome *d* differed from the cytochrome *d* of intact cells in that even in the reduced state the membrane-bound or solubilized enzyme would bind cyanide.

Finally, we have to stress that we did not find significant differences in the values of K_s, V_{max} and the pseudo-first order rate constant when different oxidation methods (air oxidation, oxidation with H_2O_2 and catalase, oxidation with a few grains of ammonium persulfate) were used for the membrane-bound or the solubilized enzyme.

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