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HEAT STABILIZATION DEPENDENCE ON REDOX STATE OF CYTOCHROME cd_1 OXIDASE FROM *Pseudomonas aeruginosa*

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SUMMARY: The irreversible thermal denaturation of cytochrome cd_1 oxidase from *P. aeruginosa* as a function of the oxidation-reduction states of its hemes was observed with a differential scanning calorimeter. Upon full reduction of the four hemes, the apparent denaturation temperature decreases by about 10° and the denaturation enthalpy decreases slightly: oxidized, 5.9 cal/gm; reduced, 5.4 cal/gm. At pH 7.5, the first order rate constants for denaturation at 90°C are: reduced, $33 \times 10^{-3} s^{-1}$; oxidized, $3 \times 10^{-3} s^{-1}$. Thus, oxidation of the hemes results in heat stabilization of the cytochrome oxidase. The activation energy for denaturation of fully reduced oxidase, 53 kcal/mol, is less than that for fully oxidized protein (73 kcal/mol).

Cytochrome cd_1 oxidase (ferrocytochrome *c*-551: oxidoreductase, EC 1.9.3.2) is a water-soluble enzyme from *Pseudomonas aeruginosa* grown anaerobically in the presence of nitrate. The protein consists of two identical subunits each of molecular weight 63,000. Each subunit has a covalently-linked heme c and a non-covalently-linked heme d (1-3). The enzyme can accept electrons from reduced azurin or ferrocytochrome *c*-551 and can convert O_2 to water by a four-electron process or nitrite to NO by a one-electron process. When reduced, hemes d_1 bind CO and CN^- cooperatively (4,5). When the oxidized protein is titrated with a reducing agent, the spectrum does not vary linearly with fraction reduced, suggesting cooperativity between the hemes (6). However, emission studies have shown the hemes to be at one end of the ellipsoidal protein molecule (7). Do changes in oxidation state have any effect on the conformation of the rest of the protein? To try to answer this question it is helpful to measure a global property such as heat stability. We have used differential scanning calorimetry to measure the kinetics and apparent temperature of denaturation of the protein in different redox states.

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EXPERIMENTAL SECTION[1]

Materials: A P. aeruginosa culture (ATCC 19429 or NCTC 6750) obtained from the American Type Culture Collection was grown on a large scale by Grain Processing Corporation, Muscatine IA. The medium used was that of Gudat, et al. (2), except that 10 mg/L $CuSO_4$ was added to the medium. The cells were grown at 32°C under an atmosphere of nitrogen. Cytochrome cd_1 oxidase was prepared at 4°C as previously described (7). The purified oxidase had an $A_{410}/A_{280} > 1.1$ (1) and an $A_{640}/A_{520} > 1.00$ (8). It gave a single band of M.W. 60,000 on a sodium dodecyl sulfate gel. Protein concentrations of solutions were determined using $E^{1\%}1$ cm (280 nm) = 18.5 (1).

Methods: Differential scanning calorimeter thermograms were recorded on a DuPont model 990 thermal analyzer modified to increase sensitivity and signal-to-noise ratio (9). The time constant for the unity-gain low bandpass filter for the Y-axis signal of the thermograms was 0.5 s for experiments at 10°/min heating rate and 10 s at 2°/min. Samples of approximately 15 µl of protein (concentration 28.6 mg/ml) in 0.06M Tris-HCl at pH 7.5 were sealed in coated aluminum hermetic pans and weighed. The reference was an equal weight of buffer solution. A stock solution of 5 mg/ml ascorbic acid dissolved in 0.05M Tris-HCl was used (appropriately diluted) to fully reduce or partially reduce the hemes. As the protein solutions were not vigorously degassed nor strict anaerobic procedures adopted, a slight excess of ascorbic acid over that needed to reduce all the hemes was added. But as the oxygen in solution was used up a partially reduced form of the oxidase was obtained. The denaturation kinetics of the partially reduced protein so obtained were intermediate between those of the fully reduced and fully oxidized proteins. To obtain the fully reduced form, a five-fold excess of ascorbic acid was added. A second heating of the samples in the calorimeter gave no endotherms. Accordingly, heat denaturation was irreversible under these experimental conditions.

Enthalpies of denaturation were calculated from the areas of the endotherms and rate constants for denaturation were calculated from the thermograms at 2° intervals as previously described (10). The vertical distance of the endotherm from the baseline at any temperature is proportional to the rate of heat flow into the sample, dH/dt, and thus is a measure of the rate of denaturation. The amount of native protein present at any temperature T can be determined by measuring the area under the peak above that temperature. The area below that temperature, designated a, is proportional to the amount of protein already heat denatured. If A represents the total peak area, then the rate constant for denaturation at temperature T is given by:
k = (heating rate) · $(dH/dt)/(A-a)$. First order kinetics were assumed.

RESULTS AND DISCUSSION

Temperatures and Enthalpies of Denaturation: Figure 1 shows typical thermograms for fully reduced, partially reduced and fully oxidized cytochrome cd_1 oxidase. Calculated enthalpies of denaturation, ΔH_d, and apparent denaturation temperatures T_d (at endotherm maxima) are listed in Table I. In general, and also for this protein, while the apparent denaturation

[1]Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

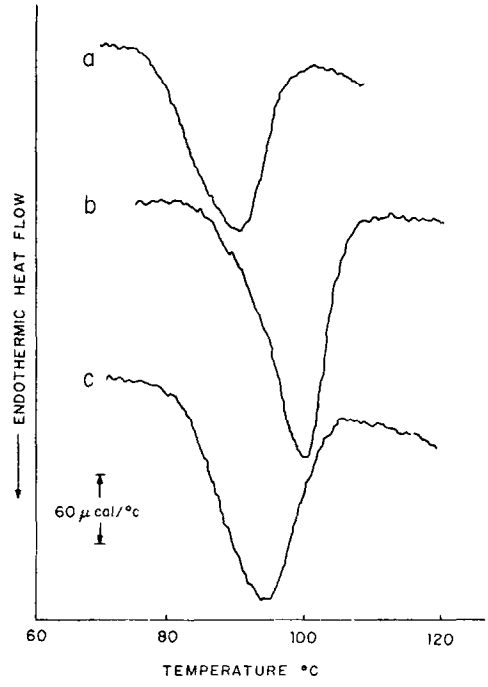

Figure 1. Denaturation thermograms of cytochrome cd_1 oxidase: (a), fully reduced, 398 μg; (b), oxidized 430 μg; (c), partially reduced, 415 μg. Heating rate, 10°/min.

temperature is a function of the rate of heating of the calorimeter cell, calculated rate constants for denaturation are not a function of heating rate (11). The true thermodynamic denaturation temperature can be inferred by extrapolation of T_d to very slow heating rates. Virtually all of the data presented here were obtained at a heating rate of 10°/min. When a heating rate of 2°/min was used, the T_d of the oxidized protein decreased by 4°. Of primary interest in the present work are the relative values of T_d measured at one heating rate (10°/min) for different oxidation states of the protein.

It is noteworthy that there is a 10° difference in T_d between the oxidized and reduced proteins. There is also a small but noticeable heat destabilization evidenced by a decrease in ΔH_d from 5.9 cal/g (∿700 kcal/mol) for the oxidized protein to 5.4 cal/g (∿650 kcal/mol) for the reduced protein. The partially reduced protein had properties intermediate between those of the fully oxidized and fully reduced proteins (Table I).

Table I: Thermal Properties of Cytochrome cd_1 Oxidase

Redox State	T_d[1]	ΔH_d[2]	Denaturation Rate Constant at 90°C	E_a
	(°C)	(cal/g)	(x 10^3 s^{-1})	(kcal/mol)
Reduced	90.5	5.4±0.2 (2)	33	53
Partially Reduced	94.5	5.8 (1)	13	53
Oxidized	100.0	5.9±0.1 (3)	3	73

[1]Determined at 10°/min heating rate. Temperature given to nearest 0.5 degree.
[2]With average deviation from mean. Number of replicates in parentheses.

Denaturation Kinetics: Rate constants for thermal denaturation of the oxidized, reduced and partially reduced proteins are plotted as a function of temperature in Fig. 2. The rate constants for denaturation of the oxidized protein were essentially independent of heating rate. Activation energies for the reduced and oxidized proteins are significantly different: 53 kcal/mol and 73 kcal/mol, respectively. For the partially reduced protein, the rate constants at high reciprocal temperatures do not coincide with those for the fully reduced protein, but lie on a line roughly parallel to them (Fig. 2). However, at low reciprocal temperatures, the rate constants for the partially reduced protein closely parallel those for the oxidized protein. This suggests that the partially reduced sample contains several species.

The endotherm for the partially reduced oxidase (Fig. 1,c) is broader than the endotherms for the fully oxidized or reduced protein. This endotherm must be the sum of the endotherms of the species C1 and C10 present (6), but cannot be the endotherm for a mixture of equal amounts of reduced and oxidized forms, since our calculations show that such an endotherm would show two peaks of heat flow. Comparison of this endotherm with those for the fully reduced (Fig. 1,a) and fully oxidized (Fig. 1,b) proteins reveals that the fraction of fully oxidized species (C1, with T_d = 100°) cannot exceed about 0.25, neither can more than a fraction of about 0.2 of the fully reduced

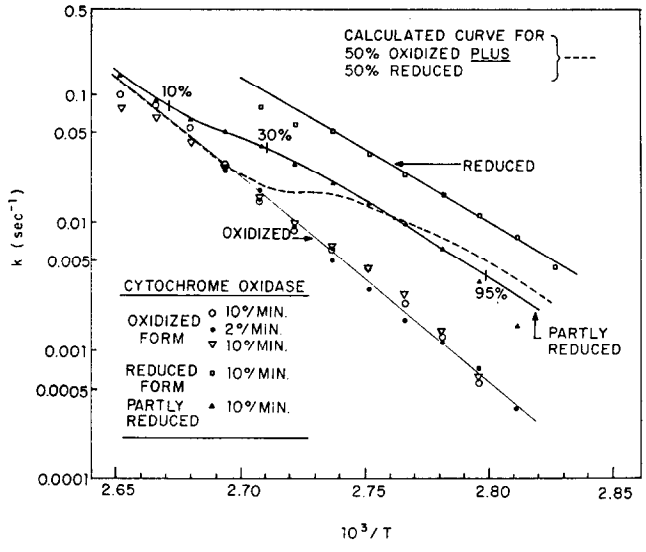

Figure 2. Arrhenius plots for thermal denaturation of fully reduced, partially reduced, and fully oxidized cytochrome cd_1 oxidase. Data are presented over the denaturation to approx. 95% denatured. For curve corresponding to partially reduced form, the amount of undenatured protein is indicated at three temperatures. Rate constants were calculated from experiments at the heating rates indicated. A calculated Arrhenius plot for a mixture of equal amounts of fully reduced and fully oxidized protein is shown as the dashed line (see text).

species (C10, with T_d = 90°) be present. Accordingly, the most probable composition of this sample, based on Fig. 3 of Blatt and Pecht (6), is that the protein is approximately one-half reduced, and that species C2 and C4 (with one and two c hemes reduced, respectively, and both d hemes oxidized) are present in largest amount. Thus species C2 (one c heme reduced) should predominate, followed in order of importance by C4 (two c hemes reduced), then C8 and C9 (three hemes reduced). A comparison of the Arrhenius plot for this sample with a calculated one for a mixture of equal amounts of oxidized (C1) and reduced (C10) protein (Fig. 2) suggests that most of the species present have heat stabilities similar to or slightly greater than C10, but that a minor amount of the protein has a heat stability similar to that of C1. (Note that the calculated Arrhenius plot for equal amounts of C1 and C10 does not coincide with that of the reduced form at lower temperatures; half the protein present has a higher heat stability (that of oxidized

form), so the rate constant for denaturation is reduced by a factor of two. At higher temperatures, only the oxidized form is present and the calculated curve coincides with the experimental one.)

CONCLUSIONS

The thermal stability of cytochrome oxidase cd_1 in the temperature range 80-110°C is dependent on the redox state of its hemes. This observation is of greater biochemical interest when related to the structure and function of the protein at physiological temperature. We have found that the circular dichroism spectra of the reduced and oxidized forms are indistinguishable at room temperature (Mitra and Bersohn, unpublished observations). However, Akey et al. (12) observed that a single crystal of oxidized cytochrome oxidase cd_1 cracked when placed in a reducing solution. Blatt and Pecht (6) have shown negative cooperativity between the c hemes, positive cooperativity between d hemes, and no apparent interaction between cd pairs. Thus, inter-subunit cooperativity is greater than intra-subunit cooperativity, would appear to be mediated by changes in tertiary or quaternary structure, and results in alterations in the dimensions of the unit cell. This very interesting behavior may be analogous to the strong inter-subunit interactions of some "4-α-helical" proteins (13), which include apoferritin monomer and some cytochromes. Because of the way the alpha helices are arranged in these proteins, inter-subunit interactions can be as strong as intra-subunit interactions.

We conclude that there is now substantial evidence for a change in molecular structure when the hemes of cytochrome cd_1 oxidase are reduced. Perhaps the most conservative interpretation of this structural change is that it is quaternary in nature, i.e., the subunits change their relative orientation. Such a change in quaternary structure could result in an altered denaturation process, i.e., differences in the kinetics and thermodynamics of randomization of tertiary structure. Cytochrome cd_1 oxidase

might thus be analogous to another four-heme protein, hemoglobin, whose quaternary structure also changes dramatically when its hemes are slightly perturbed by combination with oxygen. In each case we have a protein which is poised between (at least) two rather different quaternary states; the transition between the two is triggered by the binding of O_2 or e^-.

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