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Thermodynamics of Carbon Monoxide Binding to Monomeric Cytochrome *c'* †

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ABSTRACT: The thermodynamic parameters for carbon monoxide binding to monomeric *Rhodopseudomonas palustris* cytochrome *c'* are determined. An enthalpy change for CO(aq) binding to the cytochrome is measured directly by titration calorimetry as -6.7 ± 0.2 kcal/mol of heme, the CO binding equilibrium constant is measured at 35 °C as $(1.96 \pm 0.05) \times 10^5$ M^{-1}, and the binding equilibrium constant at 25 °C is calculated from the van't Hoff equation as $(2.8 \pm 0.1) \times 10^5$ M^{-1}. Comparison of the results to the known energetics of CO binding to dimeric cytochrome *c'*, where the CO binding site is buried in the protein interior, indicates that the heme binding site on the monomer form is, in contrast, more exposed.

The cytochromes *c'*, a class of high-spin *c*-type cytochromes, are widely distributed in the bacterial world. Although apparently functioning in bacterial electron transfer (Horio & Kamen, 1962a,b; McEwan et al., 1985), the nature of their physiological donors and acceptors are not well understood. Of particular functional interest is the fact that the cytochromes *c'* are usually isolated as dimers consisting of identical subunits, suggesting possible allosteric interactions. However, at least one example, from *Rhodopseudomonas palustris*, is isolated in monomeric form (Dus et al., 1967; Cusanovich, 1971). The high-resolution three-dimensional structure of *Rhodospirillum molischianum* cytochrome *c'* is known (Finzel et al., 1985), as well as the amino acid sequence of 12 examples (Ambler et al., 1981). On the basis of their spectral properties and their ability to bind CO, the cytochromes *c'* more closely resemble hemoglobin than the typical *c*-type cytochromes. A comprehensive literature review has been presented by Meyer and Kamen (1982).

This paper is a continuation of systematic structure–function studies on the reversible carbon monoxide binding reactions of the cytochromes *c'*. Cytochromes *c'* are especially attractive for rigorous thermodynamic analysis since their dimeric nature allows theoretical modeling of site–site interactions to be cast in the simplest possible terms. Additionally, the CO binding curves can be measured to high precision with a special thin-layer device (Dolman & Gill, 1978).

Previous work has demonstrated positive cooperativity in the CO binding curve of *Chromatium vinosum* cytochrome *c'*, the origin of which was found in a CO-linked dimer–monomer dissociation process (Doyle et al., 1986). A quan-

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titative energetic analysis of the ligand-linked dissociation scheme was reported, but the high stability of the unligated dimer state disallowed evaluation of the energetics of CO binding to the monomeric state and between the unligated monomer–dimer equilibrium. In this paper direct determination of the CO binding equilibrium properties of the monomeric form of cytochrome *c'* is made by studying *Rhodopseudomonas palustris* cytochrome *c'*, which is isolated in monomeric form (Cusanovich, 1971).

Materials and Methods

Materials. *Rhodopseudomonas palustris* cytochrome *c'* was prepared according to the method reported by Bartsch (1978) and dialyzed against 0.1 M potassium phosphate buffer. The pH of the buffer was adjusted to 7.3 by addition of either hydrochloric acid or sodium hydroxide. The enzymatic reducing system of Hayashi et al. (1973) was used to ensure complete reduction of the cytochrome sample throughout the experiments. This method of reduction is milder and more suitable for our studies than the typically used reductant dithionite. All components of the reductase system were products of Sigma Corp.

Binding Curves. Binding curves were measured with a thin-layer optical technique (Dolman & Gill, 1978), which measures changes in fractional saturation upon stepwise dilution of the partial pressure of CO above the sample solution. This method is therefore a differential one, in contrast to the usual methods for measuring ligand binding that measure absolute fractional saturation values. The dilutions were made with gaseous nitrogen by means of a dilution valve connected to the sample cell. The dilution valve generates precisely defined vales of the ligand partial pressure, which defines the activity of the ligand in solution to high precision. It should be mentioned that the number of moles of ligand in the gas phase is in large excess over the number of moles of binding sites in the sample solution (except in the case where the ligand affinity is extremely high—half-saturation values of less than 0.1 Torr). Hence correction of the ligand activity for the amount of ligand bound is unnecessary.

The partial pressure of the ligand after each stepwise dilution was calculated from

$$p_{CO}(i) = p_{CO}(0)D^i \quad (1)$$

where $p_{CO}(0)$ is the starting partial pressure of CO, $p_{CO}(i)$ is the partial pressure after the ith dilution step, and D is a constant dilution factor equal to 0.7040 ± 0.0005. Changes in the fractional saturation of CO were measured as changes in optical density at 418 nm with a Cary 219 spectrophotometer. Equilibration of the sample with each new partial pressure of CO was verified as an unchanging optical signal and required 5–10 min per dilution step. The molarity of carbon monoxide was calculated directly from partial pressure values according to Henry's law: $p_{CO} = k_H[CO]$, where k_H is Henry's constant equal to 9.130×10^5 Torr/M at 35 °C (Wilhelm et al., 1977).

Data Analysis. At each dilution in the CO partial pressure, the fractional saturation change is computed and multiplied times the parameter representing the total optical density change for complete CO ligation. The observed change in optical density, $\Delta OD(i)$, for the ith dilution step [where the CO partial pressure changes from $p_{CO}(i-1)$ to $p_{CO}(i)$] is described by

$$\Delta OD(i) = \Delta OD(t)[\theta(i) - \theta(i-1)] \quad (2)$$

where $\theta(i)$ and $\theta(i-1)$ are the final and initial values for the fractional saturation of the cytochrome with CO for the ith dilution step and $\Delta OD(t)$ is the total optical density change for complete CO ligation of the reduced protein. The data were fitted to eq 2 by an extended version of the nonlinear least-squares algorithm of Marquardt (1963).

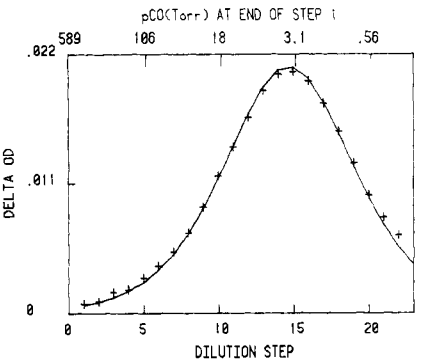

FIGURE 1: Derivative binding curve data for CO binding to *Rhodopseudomonas palustris* cytochrome *c'*. Experimental conditions were 0.1 M phosphate, 35 °C, and pH 7.3 with a starting CO partial pressure of 589 mmHg. The data are shown as optical density changes associated with designated stepwise dilutions of the partial pressure of CO. The resulting CO partial pressures are indicated along the top of the figure. The protein concentration was 0.55 mM heme, the sample thickness was 0.005 cm, the wavelength was 418 nm, and the total optical density change was 0.239 OD unit. The theoretical curve is the best fit to the data using an equation with a single equilibrium constant (eq 3). Data points are shown as crosses with vertical lengths equal to the standard deviation of a point for the fit (SEP = 0.0003 optical density unit). The intrinsic CO binding equilibrium constant was determined as $\kappa_{CO} = (1.96 \pm 0.05) \times 10^5$ M^{-1}.

Titration Calorimetry. Heats of CO ligation were measured directly in a titration microcalorimeter. The details of the calorimeter are described elsewhere (McKinnon et al., 1984). The concentration of cytochrome was determined on an aliquot of the oxidized cytochrome sample by using an extinction coefficient of 85 mM^{-1} cm^{-1} at 399 nm (Bartsch, 1978). Reduced cytochrome *c'* was prepared by adding the enzyme reducing system, followed by deoxygenation of the sample with nitrogen gas. The reduced cytochrome was then injected into a CO-saturated buffer contained in the calorimeter and the heat for complete CO ligation measured. The concentration of CO in the CO-saturated buffer was calculated from solubility data (Wilhelm et al., 1977).

Precautions to minimize heats of reaction due to solution mismatching between the sample and titrant were made as follows. First, the pH's of the sample and CO-saturated buffer were matched. Second, since many protein–ligand reactions are accompanied by proton uptake or release, phosphate buffer was used by virtue of its low proton ionization heat of 0.8 kcal/mol (Christensen et al., 1976). The proton uptake associated with CO binding to *Rps. palustris* cytochrome *c'* was determined in a separate experiment (of the pH dependence in the p_{50} value for CO) to be quite small, less than 0.1 proton taken up by the protein per CO bound. A correction heat of 0.08 kcal/mol of CO bound was therefore applied to the observed heat results.

Results

CO Binding Curve. Differential binding curve data for *Rhodopseudomonas palustris* cytochrome *c'* are shown in Figure 1. The data, which closely approximate the derivative of the binding curve, are shown as changes in optical density upon stepwise dilution of the partial pressure of CO. As expected for a single-site macromolecule, the binding data can be fit to an equation described by a single equilibrium constant. The binding partition function Q for this situation, representing

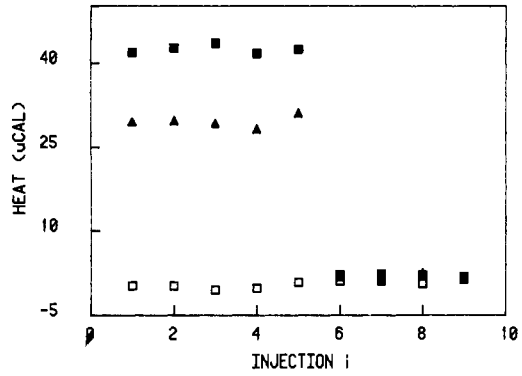

FIGURE 2: Heat of CO binding to *Rhodopseudomonas palustris* ferrocytochrome *c′* measured directly by titration calorimetry. Experimental conditions were 0.1 M phosphate buffer, pH 7.3, and either 25 (solid triangles) or 30 °C (solid squares). The data represent injections of the unligated cytochrome into a CO-saturated buffer, which leads to greater than 99% saturation of the CO binding sites. The concentration of cytochrome injected was 0.44 mM, and the injection volumes were 15 μL for the 25 °C data and 10 μL for the 30 °C data. Blanks were obtained by injecting 10-μL aliquots of 0.44 mM reduced cytochrome into buffer not containing CO and are shown as open squares along the bottom. The blank correction was less than 1 μcal. Least-squares fitting of the data gives the enthalpy change for the reaction as −6.50 ± 0.10 kcal/mol for the 25 °C data, and −6.78 ± 0.24 kcal/mol at 30 °C.

the summation of the relative concentrations of unligated and CO-ligated cytochrome, is given by

$$Q = 1 + \kappa_{CO} a_{CO} \quad (3)$$

The CO binding equilibrium constant is designated κ_{CO}, and a_{CO} is the activity of CO.

The fraction of macromolecules bound with CO, θ, is equal to the partial derivative at constant temperature of the logarithm of the binding partition function with respect to the logarithm of the ligand activity (Wyman, 1948):

$$\theta = \partial \ln Q / \partial \ln a_{CO} = \kappa_{CO} a_{CO} / (1 + \kappa_{CO} a_{CO}) \quad (4)$$

The data in Figure 1 were fitted according to eq 2 and 4, and a CO binding equilibrium constant was determined at 35 °C $\kappa_{CO} = (1.96 \pm 0.05) \times 10^5$ M^{-1}. The standard error of a point for the fit (0.0003 OD unit) approaches the sensitivity limits of the Cary 219 spectrophotometer. This shows that within the experimental limits of the measuring device the single-site nonaggregating form of the fitting equation is appropriate.

Titration Calorimetry. One major goal of the present study was to compare CO binding energetics of the monomeric cytochrome to values determined previously at 25 °C for dimeric cytochromes *c′*. However, in the present study the binding curve could not be measured at 25 °C, presumably due to very slow off-rate kinetics of the CO. An alternative approach is to measure the enthalpy change of the reaction and calculate the binding equilibrium constant at 25 °C from the van't Hoff equation

$$\kappa_{CO} = \kappa_{CO}^{\circ} \exp\left[\frac{-\Delta\bar{H}}{R}\left(\frac{1}{T} - \frac{1}{T_0}\right)\right] \quad (5)$$

where the measured equilibrium constant (at reference temperature T_0) is κ_{CO}° and the calculated equilibrium constant (at temperature T) is κ_{CO}. The molar enthalpy change for CO ligation is $\Delta\bar{H}$, R is the gas constant, and temperatures are in Kelvin units. The heat of CO binding to *Rps. palustris* cytochrome *c′* measured directly by titration microcalorimetry is shown in Figure 2 at 25 and 30 °C.

Analysis of the calorimetric experiments is based upon the enthalpy change associated with ligand binding. The enthalpy state of the macromolecule is given by the temperature derivative of the binding partition function (Gill et al., 1985), which is written explicitly as

$$Q = 1 + \kappa_{CO}^{\circ} \exp\left[\frac{-\Delta\bar{H}}{R}(\tau - \tau_0)\right] a_{CO} \quad (6)$$

τ represents the inverse of temperature ($1/T$). The molar enthalpy change in going from the unligated (reference) state of the macromolecule to a state depending on the ligand activity is then given by the partial derivative of the logarithm of the binding partition function with respect to τ:

$$-R\frac{\partial \ln Q}{\partial \tau} = \frac{\Delta\bar{H}\kappa_{CO}^{\circ} \exp\left[\frac{-\Delta\bar{H}}{R}(\tau - \tau_0)\right] a_{CO}}{1 + \kappa_{CO}^{\circ} \exp\left[\frac{-\Delta\bar{H}}{R}(\tau - \tau_0)\right] a_{CO}} = \bar{H} - \bar{H}_0 \quad (7)$$

Here $\bar{H}_0$ is the molar enthalpy of the macromolecule in the unligated reference state and $\bar{H}$ is the enthalpy at a state given by the ligand activity.

In a titration calorimeter the observed heat $q(i)$ for the ith injection is equal to the enthalpy of the macromolecule in the reaction cell at the end of the ith injection minus the enthalpy of the macromolecule in the cell at the end of the previous injection $(i - 1)$. The observed heat is equal to

$$q_i = [\bar{H}_i n_i - \bar{H}_{i-1} n_{i-1}] \quad (8)$$

where the molar enthalpy of the macromolecule at the end of the ith injection and the $i - 1$ injection is $\bar{H}_i$ and $\bar{H}_{i-1}$, respectively. The number of moles of macromolecule in the reaction cell is designated n and subscripted by the injection number i.

The calorimetric data in Figure 2 were obtained at 25 and 30 °C. The data were fitted to eq 8, and molar enthalpy changes were determined as −6.50 ± 0.10 and −6.78 ± 0.24 kcal/mol of heme at 25 and 30 °C, respectively. Within experimental error the values are the same, indicating the lack of any significant temperature dependence of the enthalpy change near 25 °C. These values include a heat of proton ionization of the phosphate buffer due to the proton uptake that accompanies CO ligation (see Materials and Methods). Thus, the heat of CO ligation is given by the average of the two measured values, minus a correction heat of 0.08 kcal/mol of CO bound for the phosphate ionization heat. The corrected average standard enthalpy change for CO ligation to *Rps. palustris* cytochrome *c′* is then equal to −6.75 ± 0.17 kcal/mol of heme.

The CO binding equilibrium constant at 25 °C was calculated as $\kappa_{CO} = (2.8 \pm 0.1) \times 10^5$ M^{-1} from the van't Hoff equation (eq 5). The equilibrium constant measured directly at 35 °C and the average enthalpy change measured by calorimetry were used in the calculation.

Discussion

The CO affinity of monomeric cytochrome *c′* is found here to be nearly 2 orders of magnitude higher than either of the dimeric cases previously studied in our laboratories (Doyle et al., 1985, 1986) and agrees with the earlier studies of Cusanovich and Gibson (1973). The standard Gibbs free energy change for the intrinsic CO binding reaction of monomeric cytochrome is equal to −7.4 kcal/mol of CO(aq), compared to the lower values of −3.1 and −5.1 kcal/mol of CO(aq) for the dimeric states of *R. molischianum* and *C. vinosum* cytochromes *c′*, respectively.

Characterization of the structural features of the monomeric and dimeric cytochromes *c′* is seen in terms of the entropic

contributions to the Gibbs free energy changes for CO binding. In the monomer case, the entropic contribution ($T\Delta S°$) to the free energy change is 0.7 kcal/mol of CO(aq). However, the entropic contribution is −4.1 kcal/mol of CO(aq) for the *C. vinosum* dimer (Doyle et al., 1986) and −7.6 kcal/mol of CO(aq) for the *R. molischianum* dimer (Doyle & Gill, 1985). The CO binding free energies of the dimer molecules therefore include a significant unfavorable entropy change that is absent in the monomer form. The unfavorable entropy change for CO binding to the dimer state is consistent with a sequestered binding site that is more accessible in the monomeric form.

The structural origin of the large differences in CO affinity by the various cytochromes *c'* is of interest since the spin-states of the three examples discussed are apparently the same on the basis of spectral properties. On the other hand, the redox potentials of these molecules are quite different (94, 18, and 14 mV for *Rps. palustris*, *C. vinosum*, and *R. molischianum*, respectively), indicating some distinctions. In terms of structural differences some observations can be made. When structural homology among the cytochromes *c'* is assumed and the structure of the *R. molischianum* molecule is used (Finzel et al., 1985), it can be seen that the side chains nearest the unligated heme face (the presumed CO binding site) are Trp-86, Met-16, and Leu-19 (3.6, 4.0, and 4.4 Å from the iron, respectively). In *Rps. palustris* the corresponding residues are Phe, Leu, and Met, and in *C. vinosum* they are Ala, Tyr, and Met. Thus, replacement of Trp by Phe or Ala should lead to substantially less steric crowding and result in a more favorable free energy change for CO ligation in both cases. The substitution of Tyr at position 16 in *Chromatium* by Leu in *Rps. palustris* may contribute to the differences in CO affinities observed between these two molecules. An alternative is that dimer formation restricts the access of CO to the heme iron. However, this is less obvious from the available structural information, since it is unclear how dimer formation perturbs the protein structure if at all. A high-resolution structure of the *Rps. palustris* molecule is needed in order to draw any definitive conclusions.

In a previous study on the CO-linked dimer–monomer process of *C. vinosum* cytochrome *c'* (Doyle et al., 1986), it was impossible to obtain a significant population of the unligated monomeric form (hence equilibria directly involving that state could not be determined), but the results indicated that a much higher CO affinity exists for the monomeric state relative to the dimer. The CO equilibrium constant for the dimer state was determined as $\kappa_D = (5.6 \pm 1.2) \times 10^3\ M^{-1}$. In the present study the monomer CO equilibrium constant, $2.8 \times 10^5\ M^{-1}$, indeed is much larger and bears out the expectation of the earlier work.

It has been pointed out that under certain solution conditions the Sephadex sizes of *Rps. palustris* cytochrome *c'* are intermediate between monomer and dimer, indicating the existence of a rapid equilibrium between the two forms (Meyer & Kamen, 1982). In the present study the CO binding curve was fitted to an equation with a single binding equilibrium constant, and a standard error of a point was obtained that approached the precision limits of the spectrophotometer. Thus within the precision limits of the measuring device and under the solution conditions used here, the molecule was essentially homogeneous.

The question arises why the *Rps. palustris* cytochrome is monomeric, whereas the *C. vinosum* molecule is dimeric. A possible explanation is revealed by inspection of the amino acid sequences of these molecules (Ambler et al., 1981) in the framework of the cytochrome *c'* crystal structure (Finzel et al., 1985). The dimer crystal structure shows two regions (portions of the A and B helices) of interaction between the subunits. Alignment of the amino acid sequences of the *Rps. palustris* and *C. vinosum* molecules at the presumed contact regions shows that the B helix in the monomer cytochrome is more hydrophilic, most likely destabilizing dimer formation. In contrast, the greater degree of hydrophobicity found in the *C. vinosum* sequence in the contact region would account for stabilization of the unliganded dimeric structure.

Registry No. CO, 630-08-0; cytochrome *c'*, 9035-41-0.

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