Noncovalent Binding of Heme Induces a Compact Apocytochrome *c* Structure†

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ABSTRACT: Mitochondrial holocytochrome *c* contains heme that is covalently attached to the protein in a reaction catalyzed by the enzyme cytochrome *c* heme lyase. In the absence of heme, apocytochrome *c*, the precursor to holocytochrome *c*, is unfolded. We find that purified apocytochrome *c* binds noncovalently to heme. Binding is accompanied by changes in the optical absorption spectrum of heme and by quenching of the tryptophan fluorescence of the protein. The affinity of apocytochrome *c* for heme, as well as the stoichiometry of binding, appears to depend on whether or not cyanide is present and on the oxidation state of heme. Under reducing conditions, in the presence of cyanide, the association appears to be 1:1, with a binding constant of about 10^7 M^{-1}. Under oxidized conditions, there may be multiple hemes bound per molecule of apocytochrome *c*. Upon binding to heme, apocytochrome *c* exhibits a mobility similar to that of holocytochrome *c* in gel filtration chromatography and velocity gradient ultracentrifugation, indicating that the heme–protein complex adopts a structure that is almost as compact as that of holocytochrome *c*. Changes in the circular dichroism spectrum of apocytochrome *c* are consistent with an increase in the α-helical content of the protein on binding heme. The compact structure of the noncovalent heme–apocytochrome *c* complex may represent an intermediate in the *de novo* folding of cytochrome *c*.

Among heme[1] proteins, the *c*-type cytochromes are distinguished by the presence of a covalent linkage between the heme and the protein. Mitochondrial cytochrome *c* is the best-studied example of this broadly distributed class of proteins. Cytochrome *c* is synthesized in the cytoplasm as apocytochrome *c*, lacking heme (Hennig & Neupert, 1981). Heme is covalently attached to two cysteines in the protein *via* thioether linkages to heme vinyl groups. This reaction, which is catalyzed by the enzyme cytochrome *c* heme lyase (Basile et al., 1980; Dumont et al., 1987; Nargang et al., 1988), appears to play a role in mitochondrial import, possibly by affecting folding of the protein (Hennig & Neupert, 1981; Nicholson et al., 1988; Dumont et al., 1991).

Even at neutral pH in the absence of denaturing agents, apocytochrome *c* adopts a noncompact structure characteristic of unfolded proteins, as demonstrated by studies of the hydrodynamic and spectroscopic properties of the protein (Stellwagen et al., 1972; Fisher et al., 1973; Cohen et al., 1974). This is in contrast to the situation for heme proteins lacking the covalent linkage to the prosthetic group. Apo forms of hemoglobin (Leutzinger & Beychok, 1981), myoglobin (Hughson et al., 1991), cytochrome *c* peroxidase (Sievers, 1978), *b*-type cytochromes (Moore & Lecomte, 1990; Feng & Sligar, 1991), and horseradish peroxidase (Strickland et al., 1968) all exhibit at least partially folded structures. Little is known about the pathways by which *c*-type cytochromes fold upon having heme attached. It has been reported that mitochondrial apocytochrome *c* does not bind to heme in the absence of heme lyase (Fisher et al., 1973; Parr & Taniuchi, 1980), but details of these experiments were not presented. The experiments described below demonstrate that purified apocytochrome *c* does, in fact, bind strongly to heme and that this binding induces formation of a compact structure.

EXPERIMENTAL PROCEDURES

Optical Absorbance Measurements. Stock solutions of apocytochrome *c*, prepared as described (Fisher et al., 1973) from horse heart holocytochrome *c* (Sigma Chemical, type VI), were obtained by dissolving the lyophilized protein in distilled water at a concentration of about 1–2 mM. All apocytochrome *c* concentrations were determined using an extinction coefficient of 0.92 mL/mg cm (Stellwagen et al., 1972). Hemin (Aldrich Chemical) was initially dissolved in 0.1 N NaOH at a concentration of 10–50 mM. The concentration was determined spectrophotometrically using an extinction coefficient of 5.84×10^4 cm^{-1} M^{-1} at 385 nm, diluted in 0.1 NaOH (Dawson et al., 1975). Heme stocks were diluted just before use either in CNP buffer (100 mM $NaH_2PO_4 \cdot 1H_2O$, 100 mM NaCN, pH adjusted to 7.5 with phosphoric acid) or in 0.1 M sodium phosphate, pH 7.5. Oxidized ribonuclease A (Sigma) was used under identical conditions to those for apocytochrome *c*.

To record the spectra shown in Figure 1, hemin was diluted from a 2.5 mM stock solution in 0.1 N NaOH to a final concentration of 10 μM in 3 mL of the appropriate buffer. Apocytochrome *c* was added from a stock solution containing 20 mg/mL. Where indicated, solutions were reduced by the addition of a few crystals of sodium dithionite (Fluka Chemical). Spectra were recorded using a cuvette with a 1-cm path length in a Hewlett Packard HP8452A diode array spectrophotometer with an integration time of 1 s.

Because of the time-dependent nature of some of the binding reactions and the longer term instability of the measured absorbances, spectrophotometric titrations were performed by preparing independent samples containing the desired

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[1] In this paper, the term heme is used to refer to iron-complexed protoporphyrin IX, regardless of oxidation state or ligands of the iron.

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concentrations of heme and apocytochrome c at timed intervals and then reading the absorbance of each sample at a defined time following mixing. Titrations requiring reducing conditions were performed in the presence of 0.25 mg/mL sodium dithionite, added from a freshly prepared 50 mg/mL stock solution immediately prior to preparing samples. The absorbances of samples with concentrations greater than 1 μM were measured using a 1-cm path length cuvette in a Hitachi 100-40 spectrophotometer. Samples with lower concentrations were measured using a 10-cm path length cuvette in a Cary 14 spectrophotometer.

Fluorescence Measurements. The fluorescence quenching curves in Figure 4 were measured by sequentially adding appropriate amounts of 0.1 mM heme stocks, in either CNP buffer or 0.1 N NaOH, to 2 mL of a 0.25 μM solution of apocytochrome c in either CNP buffer or 100 mM sodium phosphate, pH 7.5. Fluorescence measurements were performed using a 1-cm cuvette in a SPEX Industries Fluorolog 2 spectrofluorimeter, incorporating two double monochromators. The fluorimeter was operated in ratio mode, using an internal fluorescent standard. The input slits were maintained at 0.5 mm, and samples were removed from the fluorimeter immediately following each measurement to minimize photobleaching caused by ultraviolet irradiation of samples. Output slits were set to 5 mm.

Fluorescence signals were corrected for altered background levels and inner filter effects caused by the presence of heme. A background signal from samples lacking apocytochrome c and NATA was measured at each heme concentration used in the titration. This signal was well approximated as a linearly decreasing function of heme concentration. A background correction derived from a linear least-squares fit to the measured values was subtracted from each measurement of apocytochrome c or NATA[2] (*N*-acetyltryptophanamide) fluorescence. At the low protein concentrations used, the background subtraction applied in Figure 4 ranged from about 40% of the unquenched apocytochrome c signal at the lowest heme concentration to 30% at the highest heme concentration. Fluorescence of the model fluorophor, NATA, was used to correct for inner filter effects on apocytochrome c fluorescence. (The rationale for this correction is described in Results, below.) The background-corrected fluorescent signal of NATA was fit as a linear function of heme concentration (see Figure 4). The slope of this fit, expressed as a percent of unquenched NATA fluorescence, was similar to that obtained in fitting the percentage decrease of the background correction. Each background-corrected value of apocytochrome c fluorescence was divided by the NATA fluorescence at that same heme concentration, derived from the least squares fit. The apocytochrome c fluorescence was then expressed as the percent of initial, unquenched signal.

Curve Fitting. The concentration of the 1:1 heme–apocytochrome c complex [HA] formed in the reaction

$$H + A \rightleftarrows HA$$

was expressed in terms of $[H]_T$ and $[A]_T$, the total heme and apocytochrome c concentrations, and the dissociation constant K_D:

$$[HA] = (^1/_2)([H]_T + [A]_T + K_D - (([H]_T + [A]_T + K_D)^2 - 4[H]_T[A]_T)^{1/2})$$

This expression was fit to absorbance titration data using two adjustable parameters, the dissociation constant and a multiplicative factor that is related to the difference in extinction coefficient between free and bound heme. To fit fluorescence quenching data, the same fitting was performed except that the result was subtracted from 100 to obtain the percent quenching. In this case, the multiplicative factor represents the difference in fluorescence between free and bound apocytochrome c. Fitting was performed using the nonlinear least-squares feature of Sigmaplot (Jandel Scientific), weighting data points by the inverse of the standard deviation, where available. The illustrated fits converged rapidly and reproducibility from different initial estimates of the adjustable parameters. Fitted values are presented accompanied by the standard deviation of the fit in parentheses.

Gel Filtration. Samples (0.25 mL) in CNP buffer were chromatographed on a column (0.7 × 30 cm²) of Sephadex G-50 superfine equilibrated with CNP. The column was eluted at 1.1 mL/h, while collecting fractions each 11 min. Recovery of the starting absorbance of the chromatographed sample containing apocytochrome c and heme was incomplete (approximately 60% at both 410 and 277 nm). This is most likely the result of release of heme from the apocytochrome c followed by tight binding to the column packing (see text). Recovery of other samples was greater than 95%.

Velocity Gradient Centrifugation. Samples containing holocytochrome c, apocytochrome c, and heme, as indicated (total volume 0.2 mL), were layered on 4.4-mL 5–15% sucrose gradients. The gradients and the samples were prepared using CNP buffer. Gradients were centrifuged for 48 h and then fractionated from the bottom of the tube. Optical densities of the fractions were measured at 277 and 410 nm, as indicated. Since the sucrose solutions exhibited measurable absorbance at 277 nm in the absence of heme or protein, the values shown in Figure 6 were corrected by subtracting the absorbance of each fraction from a gradient to which no sample had been applied. The maximum correction was 0.04 units of absorbance at 277 nm. Recovery of initial absorbance was approximately 90% for each sample.

Circular Dichroism Spectroscopy. A cylindrical cell with a 1-mm path length was used in a JASCO Model J-600 spectrometer. A base line collected using the same cell and buffer was subtracted from each sample spectrum. Solutions of heme in the absence of protein exhibited no detectable ellipticity, and the absorbance at 200 nm of the highest concentration of heme in the samples was less than 10% of that for the protein. Data were obtained using a scanning speed of 50 nm/s, a time constant of 1 s, and a slit width corresponding to a 2 nm-bandwidth. Each spectrum displayed is the result of averaging 35 scans. Samples were diluted from stock solutions to a final composition of 1:9 water:CNP buffer.

RESULTS

Optical Absorbance Measurements. Binding of apocytochrome c to heme leads to changes in the heme optical absorption spectrum. These absorbance changes, all measured at 10 μM concentrations of heme, are shown in Figure 1. The absorption spectrum of oxidized heme in phosphate buffer is shown as the solid curve with a broad, double-lobed peak centered at 375 nm in Figure 1C. Such a spectrum is characteristic of heme aggregates (Brown et al., 1970).

Upon mixing heme and apocytochrome c in phosphate buffer, under oxidizing conditions, there is a decrease in the heme Soret absorbance to a more symmetrical peak centered

[2] Abbreviations: NATA, *N*-acetyltryptophanamide.

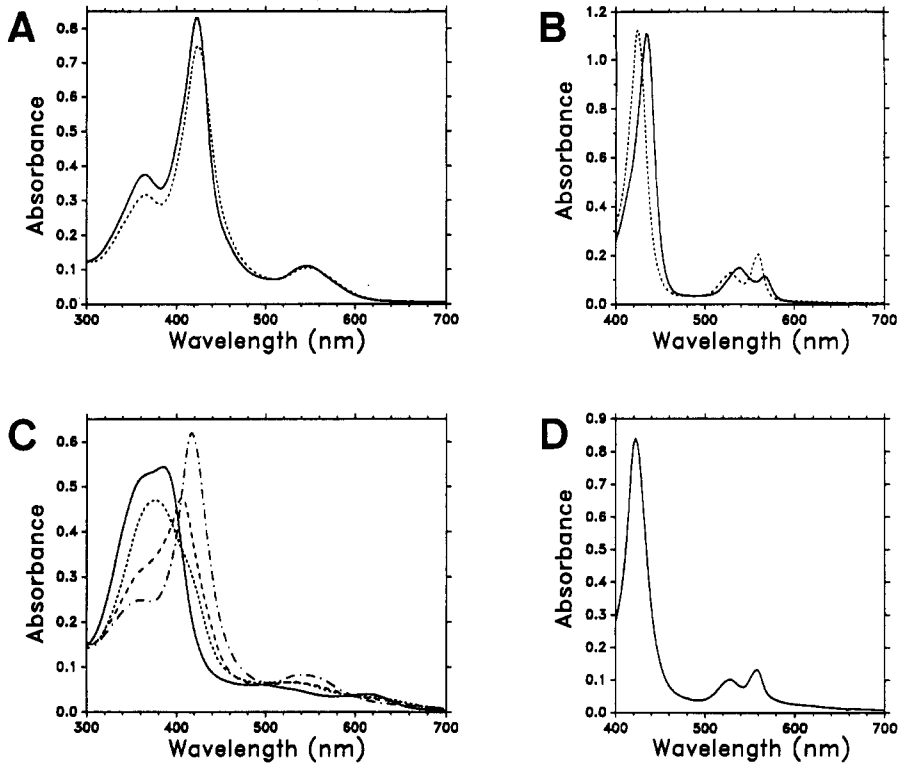

FIGURE 1: Optical absorption spectra of apocytochrome *c* and heme in the presence and absence of cyanide. All spectra were recorded at a concentration of 10 μM heme and, where present, 10 μM apocytochrome *c*. (A) Apocytochrome *c* added to heme in the presence of cyanide. Heme was diluted in CNP buffer (solid line). Apocytochrome *c* was then added (dashed line). (B) Apocytochrome *c* added to heme in the presence of cyanide under reducing conditions. Heme was diluted in CNP buffer and then reduced by the addition of sodium dithionite (solid line). Apocytochrome *c* was then added (dashed line). (C) Apocytochrome *c* added to heme in the absence of cyanide. Heme was diluted in 100 mM sodium phosphate, pH 7.5 (solid line). Apocytochrome *c* was then added. Spectra were recorded within 10 s of addition of apocytochrome *c* (short dashed line) and 20 min later (longer dashed line). Sodium cyanide was then added from a 200 mM stock (pH 7.5) to 10 mM final concentration (dotted and dashed line). The spectrum shown was multiplied by 1.05 to correct for the dilution on adding cyanide. (D) Spectrum of apocytochrome *c* and heme under reducing conditions in the absence of cyanide. Heme was diluted in 100 mM sodium phosphate, pH 7.5. Apocytochrome *c* was added and allowed to equilibrate for 20 min. The solution was then reduced by the addition of solid sodium dithionite.

at 377 nm and an increase in the broad absorbance band centered at 545 nm (Figure 1C, short dashed line). These changes occur within seconds of mixing the two components. However, the spectrum continues to change further for several minutes, eventually reaching the state indicated by the long dashed curve in Figure 1C with a Soret absorbance at 406 nm. This time-dependent transition exhibits a clear isosbestic point at 395 nm. Addition of a molar excess of apocytochrome *c* at this point leads to a further, slow increase in the 406-nm Soret band.

Attempts to reduce unbound heme in phosphate buffer led to loss of the Soret absorbance. This presumably reflects the destruction of heme that has been reported to occur upon reduction in the presence of oxygen (Lamberg & Legge, 1949a; Grasso et al., 1979). However, if dithionite is used to reduce the preformed noncovalent apocytochrome *c*–heme complex in phosphate buffer, destruction of the heme is not observed. Instead, the resulting spectrum (Figure 1D) contains sharp Soret, β, and α bands at 422, 527, and 558 nm.

To prevent aggregation of heme, many of the experiments described in this report were conducted in buffer containing sodium cyanide. Cyanide ions prevent aggregation by complexing heme. As the pK of hydrogen cyanide is about 9.2, it was necessary to use high concentrations of sodium cyanide (100 mM) to maintain a moderate concentration of ionized cyanide at pH 7.5 (Shack & Clark, 1947).

Upon complexation of heme by cyanide, the broad maximum at 375 nm (Figure 1C, solid curve) is replaced by a sharp peak at 423 nm (Figure 1A, solid curve). Addition of an equimolar amount of apocytochrome *c* in the presence of cyanide causes a small but reproducible decrease in the intensity of the Soret peak at 423 nm and of a shoulder at 365 nm. Reduction of the cyanide–heme complex in the absence of apocytochrome *c* results in the spectrum represented by the solid curve in Figure 1B, exhibiting sharpened heme Soret, β, and α bands at approximately 435, 540, and 567 nm, respectively. Addition of an equimolar amount of apocytochrome *c* causes a shift toward the blue of each of these peaks, to 425, 535, and 560 nm, accompanied by a change in the relative intensities of the α and β bands, resulting in a spectrum that closely resembles that of the reduced apocytochrome *c*–heme complex in the absence of cyanide (Figure 1D).

If cyanide is added to the preformed apocytochrome *c*–heme complex in phosphate buffer after the spectrum has stabilized (dot–dashed curve, Figure 1C), the spectrum changes to one with an overall resemblance to that of cyanide-complexed heme shown in Figure 1A, except that the Soret peak is at 417 nm, rather than at 423 nm.

The absorbance changes observed on addition of apocytochrome *c* to heme were used to study the stoichiometry of the binding reaction. The instability of the aggregation state of heme in aqueous solution made quantitative studies of optical absorbance changes impossible in the absence of cyanide, since simply mixing solutions in a cuvette was sufficient to cause small changes in absorbance. However, in the presence of cyanide, reproducible binding curves were obtained by adding increasing amounts of apocytochrome *c* to heme under reducing conditions and following the change in absorbance

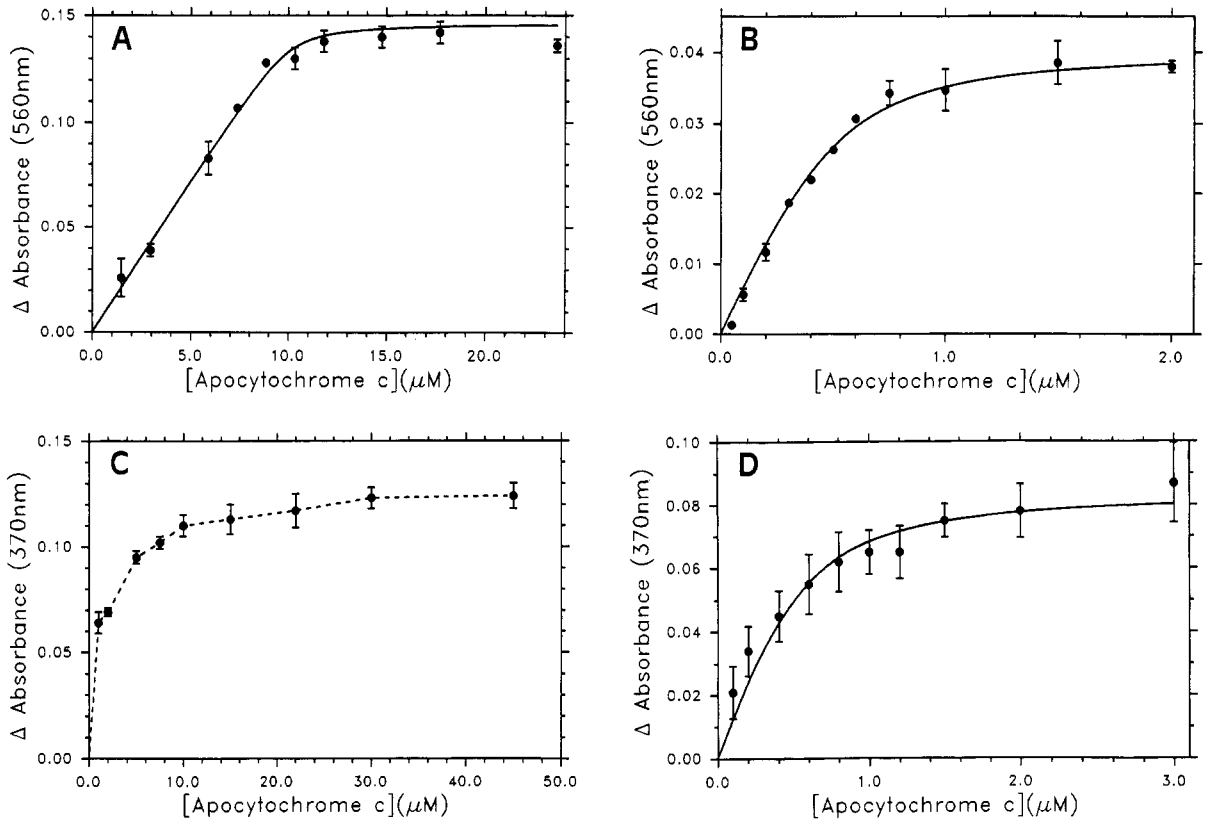

FIGURE 2: Absorbance of heme as a function of added apocytochrome *c*. Absorbances were measured using individual samples in which apocytochrome *c* and heme had been mixed in CNP buffer, containing cyanide (pH 7.5), and allowed to incubate for a fixed amount of time (see Results). (A) Samples containing 10 μM heme were prepared under reducing conditions and measured 10 min after mixing. (B) Samples containing 0.5 μM heme were prepared under reducing conditions and measured 10 min after mixing. (C) Samples containing 10 μM heme were prepared under oxidizing conditions and measured 5 h after mixing. (D) Samples containing 0.5 μM heme were prepared under oxidizing conditions and measured 30 min after mixing. The solid curves represent predictions based on best-fit dissociation constants and extinction coefficients derived from nonlinear least-squares fitting to the binding equation for 1:1 stoichiometry. The dotted line in panel C is drawn from point to point. Error bars indicate the standard deviations obtained in comparing three separate experiments. The standard deviations for points lacking error bars are less than the sizes of the symbols. In the case of panels C and D, the indicated change in absorbance reflects a decrease in extinction coefficient, relative to unbound heme.

of the α band at 560 nm. Such measurements had the advantage that most of the absorbance came from the apocytochrome *c*–heme complex, with relatively little interfering contribution from free heme (see Figure 1B). Under these conditions, the absorbance of heme- and apocytochrome *c*-containing solutions declined slowly with time, presumably because of a combination of oxidation of the solution and degradation of heme. Thus, the binding assays were performed by mixing solutions containing the desired constituents, adding dithionite, and, then, measuring the absorbance after 10 min, rather than through a successive additions to a single solution. Measurements of the kinetics of the reaction confirmed that the binding-related absorbance changes had stabilized by this time. When binding is assayed at relatively high concentrations of reactants (10 μM heme), the resulting curve (Figure 2A) appears to be a simple titration of binding sites, indicating that the concentrations of reactants are considerably higher than the dissociation constant. The position of the sharply curved region of the titration is indicative of a 1:1 binding stoichiometry. The calculated binding curve in Figure 2A was drawn assuming 1:1 stoichiometry and using a fixed value of 9×10^{-8} M for the dissociation constant, measured from the absorbance titration at 0.5 μM heme, described below. A value of 1.46 (±0.02) × 10^4 cm^{-1} M^{-1} for the change in extinction coefficient of heme on binding to apocytochrome *c* was obtained by nonlinear least-squares fitting of the data in Figure 2A with the fixed dissociation constant.

Measurement of binding at high reactant concentrations is optimal for determination of the binding stoichiometry. However, in order to obtain a more reliable estimate of the dissociation constant for the heme–apocytochrome *c* reaction, a titration was performed at a lower concentration of heme (0.5 μM), in order to expand the curved region of the binding isotherm that determines the binding affinity. For these measurements, a cuvette with a 10-cm path length was used to increase the absorbance to a measurable range (Figure 2B). Fitting of this data to a 1:1 binding stoichiometry yielded a dissociation constant of 9 (±2) × 10^{-8} M and an extinction coefficient of 8 (±3) × 10^3 cm^{-1} M^{-1} for the change in absorbance on binding. The discrepancy between the fitted extinction coefficients depending on concentration is reproducible (see error bars, Figure 2A,B) and appears to be the result of deviations from Beer's law in the absorbance of the heme–apocytochrome *c* complex (not shown).

To allow comparison with fluorescence measurements described below, some binding measurements were performed by successively adding increasing amounts of heme to a solution of 1 μM apocytochrome *c* (Figure 3). Performed in the presence of cyanide, under reducing conditions, this leads to an increase in optical density that exhibits two phases. The first phase represents the absorbance due to increasing concentrations of the heme–apocytochrome *c* complex. The second phase, at levels of heme that are in stoichiometric excess compared with apocytochrome *c*, is the Beer's law increase proportional to increasing concentration of unbound

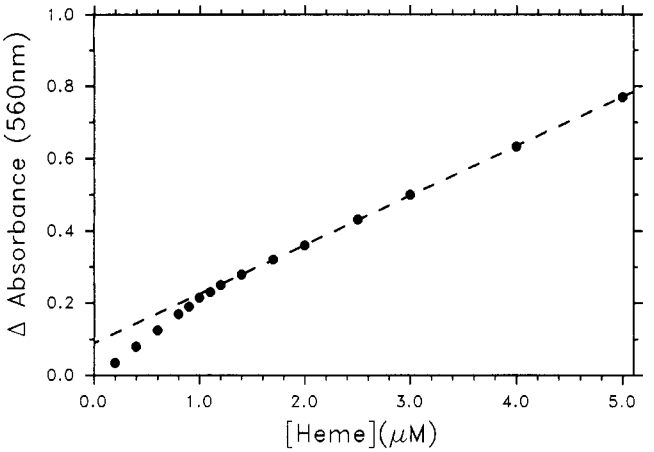

FIGURE 3: Absorbance of an apocytochrome *c*–heme mixture as a function of added heme. Increasing amounts of heme were sequentially added to a 1 μM solution of apocytochrome *c* in CNP buffer in the presence of 0.25 mg/mL sodium dithionite. The dashed line represents a linear least-squares fit to a straight line, using the points at greater than 1.2 μM heme.

heme. The intercept at the vertical axis of a straight line fit to the second phase of the plot represents the excess absorbance due to formation of the heme–apocytochrome *c* complex. This intercept occurs at a value of approximately 10^4 cm^{-1} M^{-1}. The breakpoint where the slope approaches the Beer's law value occurs at approximately 1 μM heme, consistent with a K_D of less than 1 μM for the interaction.

The absorbance changes associated with apocytochrome *c* binding to heme were also examined in oxidized solutions in the presence of cyanide. In this case, we measured the decrease in absorbance at 370 nm on binding to apocytochrome *c*. This is the wavelength at which there is the greatest percentage difference between unbound heme and heme–apocytochrome *c* complex in the presence of cyanide. However, the observed changes are small as a percentage of total heme absorbance and indicate disappearance of free heme rather than formation of any particular complex. Furthermore, when the measurements were performed at 10 μM heme, the absorbance changes took a long time to stabilize. Following an initial phase with a time constant of a few minutes, the absorbance continued to change slowly for much longer times. Thus, samples were incubated for 5 h before measuring the absorbance. By this time, the absorbance had stabilized.

The binding curve observed under oxidizing conditions in the presence of cyanide could not be fit to a model based on 1:1 stoichiometry. Binding of apocytochrome *c* to heme, as indicated by the absorbance changes (Figure 2C), did appear to reach a partial plateau at about 1:1 stoichiometry, but the shape of the curve below this level is not consistent with simple titration of a single class of identical binding sites. The substantial change in absorbance at low ratios of apocytochrome *c* to heme is consistent with binding of multiple hemes per protein. The fact that the absorbance continues to increase up to about 10 μM apocytochrome *c* suggests that there is a shift of heme binding from lower to higher affinity sites as the sites become available in samples with higher apocytochrome *c* concentrations. Such a kinetic redistribution of heme binding also provides an explanation for the very slow changes in absorbance of individual samples in this experiment. If the association rates for the two sites are similar, heme could be binding to both low- and high-affinity sites upon initial mixing. During the subsequent incubation, heme would be expected to dissociate more rapidly from the lower affinity sites, eventually ending up bound primarily to the high-affinity sites. Consistent with this scenario, binding of heme to apocytochrome *c* under identical conditions but at lower concentrations (0.5 μM; Figure 2D) did not exhibit the very slow kinetic phase seen at higher concentrations. The absorbance change measured from individual samples containing various ratios of apocytochrome *c* to heme could be measured following 30 min of incubation. The data at the low concentrations could be fit to a model based on a single binding event with a K_D of 1.4 (±0.7) × 10^{-7} M and an extinction coefficient of 1.7 (±0.1) × 10^4 cm^{-1} M^{-1}. However, even at this low heme concentration, where binding to low-affinity sites on the protein should be suppressed, the absorbance change at low apocytochrome *c* concentrations increases slightly more steeply than the single-site model would predict.

Fluorescence Measurements. Proximity of the heme to Trp59 of holocytochrome *c* leads to greater than 95% quenching of the tryptophan fluorescence compared with that of guanidine-denaturated holoprotein (Tsong, 1974). Thus, binding of heme to apocytochrome *c* was investigated by measuring quenching of the intrinsic fluorescence of the protein by heme.

The intensity of tryptophan fluorescence from apocytochrome *c* is approximately the same as that of an equal concentration of the model compound NATA, increasing concentrations of heme quench the apocytochrome *c* fluorescence in a nonlinear fashion (Figure 4). Addition of heme to NATA, under identical conditions, causes a smaller decrease in fluorescence that is approximately linear with heme concentration and can be attributed primarily to inner filter effects [see Cantor and Schimmel (1980)]. Thus, each value of apocytochrome *c* fluorescence has been corrected for inner filter effects and dilution using the measured fluorescence of NATA determined in an identical series of heme titrations. The background signal observed in the absence of any apocytochrome *c* or NATA also depended on the heme concentration, presumably because of subtle changes in the light scattering and optical absorption of the solutions. To correct for these variations, the background signal was measured at each individual heme concentration.

To minimize the effects of heme aggregation in fluorescence measurements, concentrations of apocytochrome *c* and heme were kept to the minimum necessary for detection, about 0.25 μM apocytochrome *c*, under the conditions used. The optical absorbance spectrum of heme under these conditions, measured using a 10-cm path length, is similar to that shown in Figure 1C (results not shown), indicating that most of the heme is not monomeric (Brown et al., 1970). Apocytochrome *c* fluorescence was quenched 50% at a heme concentration of about 0.1 μM. The fluorescence quenching data were fit to a 1:1 heme:apocytochrome *c* binding model according to methods described in Experimental Procedures. The fit (shown as the solid line in Figure 4A) yielded a dissociation constant of 5.1 (±0.7) × 10^{-8} M and a maximal level of 95 (±1)% quenching. When similar fluorescence quenching experiments were performed using 1 μM apocytochrome *c* and correspondingly higher concentrations of heme, the dissociation constants derived from the quenching curve were higher, presumably because of competition between higher aggregation states of heme and binding to the protein.

In the presence of cyanide, apocytochrome *c* fluorescence was quenched 50% at a heme concentration of about 0.5 μM. Thus, 0.5 μM represents an upper limit for the dissociation constant of the heme–protein interaction. An attempt to fit this quenching curve using a 1:1 binding curve is shown by the curve in Figure 4B. This fit yielded a dissociation constant

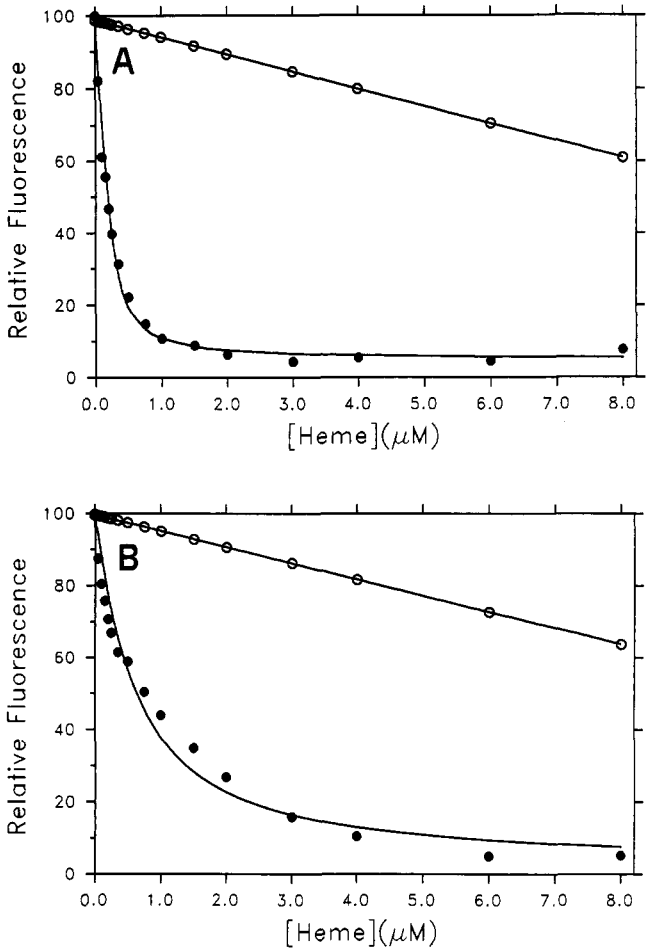

FIGURE 4: Fluorescence quenching of apocytochrome *c* by heme in phosphate buffer. Filled circles: fluorescence of apocytochrome *c* (0.25 μM) following background subtraction and correction for the inner filter effect. Open circles: fluorescence of NATA (0.25 μM) following background subtractions (see Experimental Procedures). The straight lines represent first-order linear fits to the NATA fluorescence data. The curved lines associated with the filled circles show the calculated fit to the quenching data on the basis of an assumption of 1:1 binding stoichiometry (see text). The excitation wavelength was 290 nm. Emission was detected at 345 nm. Fluorescence of NATA and apocytochrome *c* were each set to 100% in the absence of heme. The abscissa refers to total heme concentration in the cuvette. (A) Fluorescence was determined in phosphate buffer under oxidized conditions. (B) Fluorescence was determined in CNP buffer, containing cyanide, under oxidizing conditions.

of 4.8 (±0.7) × 10^{-7} M and a maximum quenching of 98 (±4)%. However, the shape of this curve appears to deviate substantially from the trace of the data points, descending more steeply at low heme concentrations and less steeply at higher concentrations, raising the possibility that the observed behavior could be the result of two or more binding events, with dissociation constants above and below that derived from fitting to a single class of sites.

Lack of information concerning the relative orientations and rotational mobilities of the tryptophan and heme, as well as the possibility of multiple binding modes, makes it difficult to estimate the precise distance between chromophores using the relations describing nonradiative energy transfer. However, given the similarity between the magnitudes of the quenching seen in the heme–apocytochrome *c* complex, compared with that of holocytochrome *c*, the distance between the heme and the tryptophan in the most highly quenched complexes could be as close as 10 Å, as seen in the X-ray structure of holocytochrome *c* [see Bushnell et al. (1990) and Jeng and Englander (1991)].

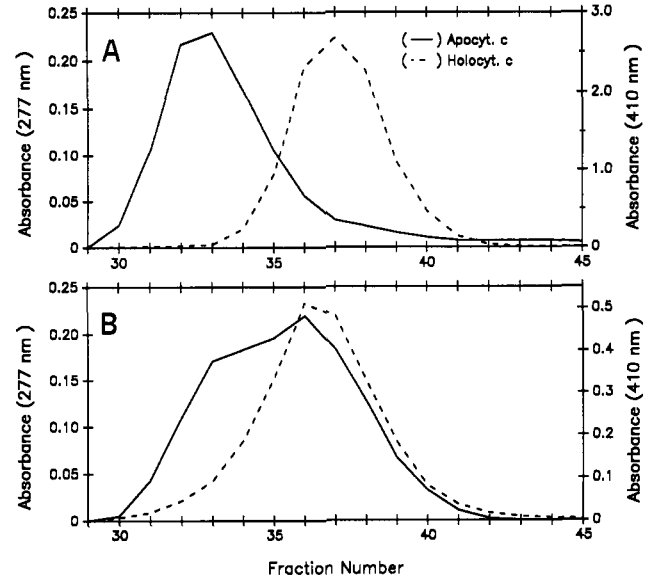

FIGURE 5: Gel filtration chromatography of apocytochrome *c* plus heme. (A) Chromatography of holocytochrome *c* and apocytochrome *c* in the absence of heme. The two profiles are the result of separate experiments. Elution of holocytochrome *c* (80 μM) was monitored by measuring absorbance of fractions at 410 nm (scale at right). Elution of apocytochrome *c* (85 μM) was followed by monitoring absorbance at 277 nm (scale at left). (B) Chromatography of apocytochrome *c* (85 μM) plus heme (80 μM). Solid line: absorbance at 277 nm. Dashed line: absorbance at 410 nm.

Gel Filtration. As an additional test of heme binding to apocytochrome *c*, a mixture of heme and apocytochrome *c* (85 μM of each) was subjected to gel filtration chromatography on Sephadex G-50 in CNP buffer. Because of its unfolded conformation, apocytochrome *c* migrates with an apparent molecular weight of 30 000, while holocytochrome *c* migrates as would be expected from its actual molecular weight of approximately 12 500 (Fisher et al., 1973; Dumont & Richards, 1984) (see Figure 5A). In the absence of protein, heme binds strongly to the column packing, forming a narrow greenish band that is only eluted after washing with several column volumes, as reported previously (Fisher et al., 1973). In performing gel filtration of apocytochrome *c* and heme, the optical absorbance of column fractions was measured at both 410 nm, where heme absorbs but apocytochrome *c* does not, and at 277 nm, where the two molecules have approximately equal molar extinction coefficients in CNP buffer. When an apocytochrome *c*–heme mixture is loaded on the column (Figure 5B), the major peak of absorbance at 410 nm elutes at approximately the same position as holocytochrome *c*. This demonstrates that much of the heme remains bound to the protein during passage through the column and that the protein with bound heme is more compact than unbound apocytochrome *c*. The possibility that slower migration of the protein in the presence of heme might be due to interaction of the heme-containing complex with the column packing is inconsistent with the results of sedimentation velocity measurements, described below. The peak of the absorbance profile at 277 nm in Figure 5B is coincident with the peak at 410 nm, but the 277-nm profile exhibits a broad shoulder extending to smaller elution volume of unbound apocytochrome *c*. This shoulder may consist of apocytochrome *c* from which the heme has dissociated and bound to the column packing. At the concentrations used, based on the binding constant determined as described above, essentially all of the protein should have initially bound heme.

Velocity Gradient Centrifugation. Heme binding to apocytochrome *c* was also examined by sedimentation velocity

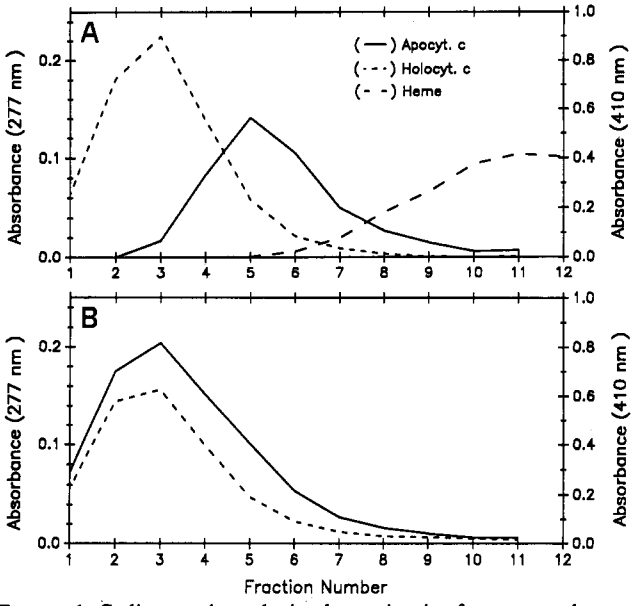

FIGURE 6: Sedimentation velocity determination for apocytochrome *c* plus heme. (A) Apocytochrome *c* (85 μM), holocytochrome *c* (80 μM), and heme (80 μM) were applied to separate 5–15% (w/w) sucrose gradients and centrifuged at 420000g_{max} for 50 h at 10 °C. The absorbance of the fractions was measured at 277 nm (apocytochrome *c*, solid line) and 410 nm (holocytochrome *c*, short dashed line, and heme, long dashed line). (B) A mixture of apocytochrome *c* (85 μM) and heme (80 μM) was applied to a gradient and centrifuged as for panel A. The absorbances of the fractions were determined at 410 nm (dashed line) and 277 nm (solid line).

experiments using density gradients. In the presence of an equal concentration of apocytochrome *c* (80 μM), essentially all the heme that was applied to a gradient sedimented at a velocity similar to holocytochrome *c* (Figure 6), with a sedimentation coefficient of 1.8–2.0 S (Stellwagen et al., 1974; Margoliash & Lustgarten, 1962), rather than to apocytochrome *c*, with a sedimentation coefficient of 1.2 S (Stellwagen et al., 1972). This increase in the sedimentation velocity of apocytochrome *c* on binding heme can not be due to protein aggregation, since the heme–protein complex exhibits a smaller hydrodynamic radius in gel filtration experiments.

Heme that was applied to gradients in the absence of protein remained in the top fractions, aside from diffusional spreading. The lack of heme in lower fractions of these gradients confirms that the molecule is not extensively aggregated in the cyanide-containing buffer. Similarly, the lack of apocytochrome *c* in the bottom fractions of these gradients (and others sedimented for shorter times; not shown) demonstrates that apo- and holocytochromes *c* remain monomeric under these conditions.

Circular Dichroism Spectroscopy. The major feature of the CD spectrum of apocytochrome *c* is a negative peak at 200 nm typical of "random coil" (Stellwagen et al., 1972; Fisher et al., 1973). The dichroic spectrum of holocytochrome *c*, on the other hand, exhibits bands at 210 and 222 nm, consistent with the significant α-helical content of the native structure. Figure 7 shows the change in the circular dichroism of apocytochrome *c* on binding of heme. With increasing heme concentration, a progressive increase is seen in the negative ellipticity at 220 nm accompanied by a decrease in the 200-nm band, consistent with increasing helical content of the protein on binding of heme. No significant additional changes were observed as the heme concentration was raised above 12 μM heme (not shown).

Comparison of Apocytochrome c with Oxidized Ribonuclease. We wished to determine whether the interaction of heme with apocytochrome *c* reflected a general property of unfolded proteins, perhaps mediated by the hydrophobicity of the molecules involved, or whether the interaction was specific for this particular protein. For this purpose, it would be useful to be able to compare the affinity of apocytochrome *c* for heme with that exhibited by other unfolded proteins. Unfortunately, the unique properties of apocytochrome *c*, specifically its existence as a soluble monomer in an unfolded state under normal buffer conditions at neutral pH and in the absence of denaturants, make it difficult to find other proteins that would be unfolded under the mild conditions used in the binding assays described above. One example of a protein with a stably unfolded form is oxidized ribonuclease A (Hirs, 1967). This protein is similar in charge and size to apocytochrome *c*. We have confirmed that oxidized ribonuclease A exhibits a significant shift in its elution from a G-50 Sephadex gel filtration column, compared with native ribonuclease A, indicative of a significantly larger radius of gyration than that of the native protein (results not shown). However, when oxidized ribonuclease was preincubated with heme in the presence of cyanide and then subjected to gel filtration under conditions identical to those used for gel filtration of apocytochrome *c*, less than 5% of the added heme co-eluted with the protein, as indicated by the absorbance at 410 nm. This was true even when the heme and the ribonuclease were present at twice the concentrations used for the experiments with apocytochrome *c* shown in Figure 5. Following the preincubation with ribonuclease, most of the heme was bound to the bed of the column, eluting after washing with several column volumes. Furthermore, no absorbance changes were seen upon incubating ribonuclease with heme, either in the presence or absence of cyanide. Ribonuclease A contains no tryptophan residues, but upon incubation with heme, the decrease in tyrosine fluorescence of the protein was no greater than the decrease in the fluorescence of *N*-acetyltyrosinamide caused by inner filter effects under identical conditions (results not shown).

DISCUSSION

We have used several independent methods to demonstrate that heme binds noncovalently to apocytochrome *c*. The tryptophan fluorescence and circular dichroism spectra of apocytochrome *c* are substantially altered upon mixing with heme. The optical absorption spectrum of heme is affected by the presence of apocytochrome *c*. Apocytochrome *c* and heme were found to cofractionate in gel filtration chromatography and sedimentation velocity measurements. The detection of a noncovalent interaction between heme and apocytochrome *c* that persists in the presence of high concentrations of cyanide appears to contradict a previous report that failed to find any such interaction (Parr & Taniuchi, 1980). However, the existence of a noncovalent interaction between heme and apocytochrome *c* is consistent with the results of Przywarska-Boniecka and Ostropolska (1982), who reported that apocytochrome *c* binds tetrasulfonated pthalocyanines, inducing changes in the conformation of apocytochrome *c* similar to those that we observed. Upon prolonged incubation of the pthalocyanine–apocytochrome *c* complex with a molar excess of heme, the bound pthalocyanine was replaced by heme (Przywarska-Boniecka & Ostropolska, 1982).

Formation of a stable noncovalent complex between heme and apocytochrome *c* is also consistent with evidence from X-ray structures of holocytochromes *c* that there are extensive interactions between heme and various amino acid residues in widely distributed regions of the protein (Bushnell et al.,

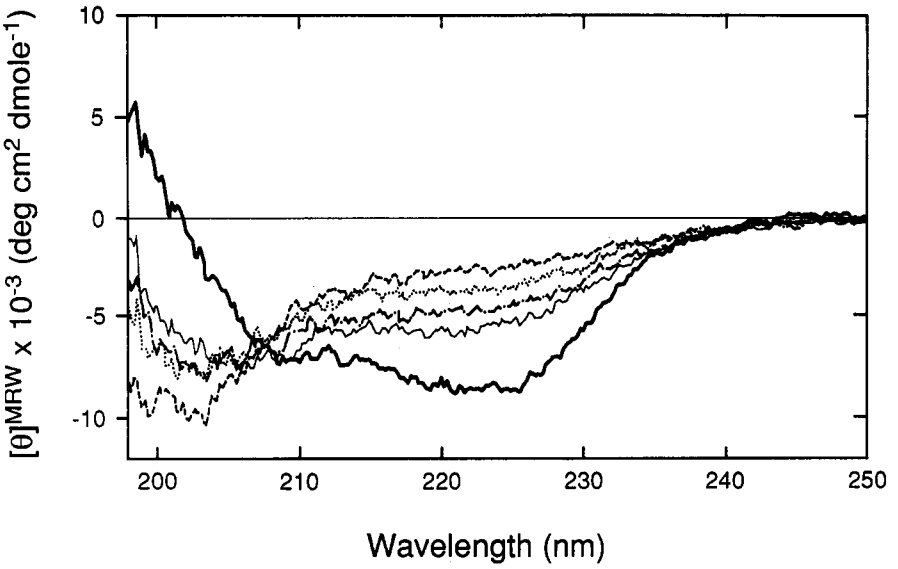

FIGURE 7: Circular dichroism spectra of apocytochrome c, holocytochrome c, and apocytochrome c with heme. Mean residue ellipticity $[\theta]^{MRW}$ is plotted on the ordinate of the following spectra: (▬) holocytochrome c, (– – –) apocytochrome c, (• • •) apocytochrome c + 4 μM heme, (• – • –) apocytochrome c + 8 μM heme, and (—) apocytochrome c + 12 μM heme. The apocytochrome c concentration was 8.5 μM.

1990). Assuming that the noncovalent binding of heme to apocytochrome c involves the same contact points that are seen in the intact protein, the highest of the observed binding constants suggests noncovalent heme–protein interactions could contribute as much as 10 kcal/mol free energy to the folded structure, comprising a large fraction of the total free energy of folding.

Binding Affinity and Stoichiometry. The results of optical absorbance measurements in the presence of cyanide under reducing and oxidizing conditions and of fluorescence quenching measurements under oxidizing conditions in the absence of cyanide all demonstrate the existence of a relatively high-affinity interaction between apocytochrome c and heme with a dissociation constant on the order of 10^{-7} M. Under reducing conditions in the presence of cyanide, optical absorbance changes on binding of apocytochrome c to heme saturate at a binding stoichiometry of 1:1, even when the reactants are present at concentrations much greater than the K_D. However, under oxidizing conditions in the presence of cyanide, fluorescence quenching and absorbance measurements lead to binding curves that, while consistent with the existence of a high-affinity interaction, also indicate the existence of at least one additional class of lower affinity sites. Unfortunately, it has not been possible to compare the fluorescence measurements obtained with cyanide under oxidizing conditions with similar measurements under reducing conditions because of the high concentrations of dithionite necessary to keep heme reduced in the presence of cyanide. Oxidation of the dithionite during the measurements causes significant changes in the optical density of the solutions, resulting in artifactual changes in the apparent fluorescent intensity.

Differences in the apparent binding stoichiometry associated with the oxidation state of heme could result from differences in the actual binding under the two sets of conditions, from differences in the aggregation state or affinities for ligands of the unbound heme, or from differences in the assays. Under reducing conditions, we measured the appearance of the 560-nm α band characteristic of the heme–apocytochrome c complex. Under oxidizing conditions, we measured either a reduction in the 370-nm absorbance of free heme or quenching of the tryptophan fluorescence on formation of the complex. It is possible that low-affinity interactions contribute to the quenching and modification of the free-heme absorbance but do not result in formation of the specific 560-nm absorbance band of the complex. Although the fluorescence quenching measurements indicate the existence of only one high-affinity site in the absence of cyanide, such data can not be used to rule out other binding stoichiometries, since the high-affinity binding causes essentially complete quenching.

Apocytochrome c Conformational Change. At high concentrations of reactants, binding of heme induces a structural change in apocytochrome c: (1) Fluorescence of the tryptophan in apocytochrome c is quenched almost as efficiently by the noncovalently bound heme as by the covalently bound heme in holocytochrome c. In contrast, fluorescence of this tryptophan is only 10% quenched, compared with that of free tryptophan in holocytochrome c denatured by urea and low pH (Tsong, 1974). (2) Heme binding causes condensation of apocytochrome c from its normal, relatively extended conformation to a form that is approximately as compact as holocytochrome c, as assayed by gel filtration and sedimentation velocity measurements. (3) The circular dichroism spectrum of the heme–protein complex indicates that the α-helical content of the protein is intermediate between that of apocytochrome c and that of holocytochrome c. The combination of compactness and partial secondary structure is characteristic of partially folded protein structures that have been referred to as molten globules or collapsed forms. Such structures have been detected as transient species in kinetic studies of protein refolding and as stable species in the presence of high salt concentrations under acid conditions (Dill & Shortle, 1991).

We have presented evidence that the stoichiometry of heme binding to apocytochrome c may not be 1:1 under oxidizing conditions in the presence of cyanide. Since the studies of the apocytochrome c conformational change on binding heme were performed under these same conditions, this raises the question of whether the observed conformational changes in apocytochrome c are the result of higher or lower affinity binding. In fact, the following three observations support the idea that the conformational changes are associated with high-affinity binding: (1) The virtually complete quenching of apocytochrome c fluorescence at low concentrations of heme (Figure 4A) indicates that, at least in the absence of cyanide, the high-affinity binding event brings the heme close to the tryptophan. (2) The changes in circular dichroism of

apocytochrome *c* associated with heme binding appear to saturate near a 1:1 ratio of reactants. (3) The gel filtration and sedimentation velocity experiments were performed at 1:1 heme:apocytochrome *c* ratios over periods of many hours. Under these conditions, depending on the dissociation rates and the actual number of different sites, a large fraction of the heme would be expected to be bound to the highest affinity class of sites, both because of the initial binding distribution and because of redistribution during incubation, as indicated by the slow changes in absorbance described under Results. If high-affinity binding did not result in formation of the more compact structure, we would expect to find at least a similarly large fraction of the heme–protein complex behaving like apocytochrome *c* in the gel filtration and sedimentation velocity measurements. However, in the gel filtration profile of the heme–apocytochrome *c* complex shown in Figure 5, very little of the complex behaves like apocytochrome *c*. The absorbance at 410 nm of the fractions which co-elute with apocytochrome *c* is only about 5% of the absorbance of the peak that coelutes with holocytochrome *c*.

The complex behavior of the fluorescence quenching under oxidizing conditions in the presence of cyanide suggests that under these conditions the highest affinity binding event causes only a partial quenching of the tryptophan signal. The presence of one or two cyanide ligands may prevent heme from gaining the close access to the tryptophan that is possible in phosphate buffer, where the iron would be expected to be coordinated by more loosely bound hydroxyl groups (see below). Cyanide is capable of displacing one of the heme ligands in native holocytochrome *c* and reduces the extent of fluorescence quenching by heme in guanidine hydrochloride–denatured holocytochrome *c* (Tsong, 1974).

Coordination of the Heme Iron. In the absence of cyanide, the marked changes in the heme absorption spectrum on adding protein suggest that groups from the protein are serving as ligands of the heme iron, as would be expected from studies of heme ligands in denatured holocytochrome *c* (Muthukrishnan & Nall, 1991). Further support for the involvement of the protein groups in liganding the heme iron comes from the observation that both cyanide binding and apocytochrome *c* binding protect heme against oxidative damage in the presence of sodium dithionite.

The strong similarity between the wavelengths and relative peak heights of the absorption maxima of the reduced heme–apocytochrome *c* complexes formed in the presence and absence of cyanide indicates that cyanide is not retained as the only heme ligand upon binding to apocytochrome *c*. Furthermore, under reducing conditions, in the presence of cyanide, the absorption spectrum of the heme–apocytochrome *c* complex is significantly different from that of heme in the absence of apocytochrome *c*. This change in the absorbance spectrum of reduced heme on binding to apocytochrome *c* is of comparable magnitude to changes seen upon displacement of heme-bound cyanide by other small ligands (Lamberg & Legge, 1949b). Under oxidizing conditions, the absorbance spectra of the heme–apocytochrome *c* complex show only moderate changes in extinction coefficient, compared with those of the unbound heme in cyanide. However, substitutions of ligands in model compounds can under some conditions cause virtually no change in the oxidized absorption spectrum. For example, substitution of pyridine for one of the cyanide ligands in oxidized dicyanide-complexed heme causes almost no change in the wavelength maximum or extinction coefficient of the 548-nm absorption band (Lamberg& Legge, 1949b). Similarly, binding of oxidized heme dicyanide to apomyoglobin causes only minor perturbations of the strengths of heme absorbance bands [see Kawamura-Konishi et al. (1988)].

The time-dependent two-state transition evident in the absorption spectrum following the mixing of heme and apocytochrome *c* in the absence of cyanide could represent either a slow protein-folding event, perhaps related to the process by which Met 80 becomes a heme ligand during the refolding of holocytochrome *c* (Ridge et al., 1981), or a slow rearrangement of the heme environment in the bound complex. A similar kinetic phase is seen in the presence of cyanide, both under reducing and oxidizing conditions, suggesting that the time dependence is not related to disaggregation of heme multimers. Since the time required to achieve a stable absorbance does not appear to be a strong function of concentration of the reactants, the rate-determining step appears to consist of a slow rearrangement of the prebound complex (data not shown). Unlike, the absorbance measurements, fluorescence quenching does not exhibit a kinetic phase that can be observed at a time scale limited by the tens of seconds it takes to mix samples and insert them into the fluorimeter, even at the lowest concentrations used. Taken together, these observations suggest that binding of apocytochrome *c* to heme occurs by a two-step pathway in which an initial interaction brings the heme into close proximity to the fluorescent tryptophan of apocytochrome *c*. This initial interaction alters the environment of heme, as seen from the rapid optical changes seen in phosphate buffer and from the effects of cyanide on the rapid fluorescence changes, but may not involve replacement of the groups coordinating the iron. The second step, inferred from the slower optical absorbance changes, could be a rearrangement of the structure of the heme–protein complex that replaces one of the original ligands of the heme iron with chemical groups from the protein. Retention of one bound cyanide is consistent with the observation that cyanide is capable of replacing Met 80 as one of the heme ligands in native holocytochrome *c*, whereas replacement of His 18, the second ligand in the native protein, requires strongly denaturing conditions (Tsong, 1974).

In two other proteins, myoglobin and the hemoglobin α chain, the sequence of events during binding of cyanide-complexed heme to the apoprotein has been explained in terms of protein structural changes as well as changes in the immediate environment of heme. In the case of myoglobin, the sequence of events is similar to that proposed here—an initial binding event and change in protein structure followed by a slower substitution of a protein ligand for cyanide, with a first-order rate constant on the order of 10^{-2} s^{-1} (Kawamura-Konishi et al., 1988). In the hemoglobin subunit, the change appears to occur in reverse order, with a rapid rearrangement of the heme environment followed by slower adjustments in overall protein conformation (Leutzinger & Beychok, 1981).

Significance of Heme Binding to Apocytochrome c. The experiments that we have performed with oxidized ribonuclease suggest that the heme binding we observe for apocytochrome *c* is not a general property of unfolded proteins, even those which, like ribonuclease, contain multiple histidine residues. However, it seems likely that at least a portion of the energy of binding of heme to apocytochrome *c* results from hydrophobic interactions, given the membrane active and hydrophobic properties of apocytochrome *c* (Dumont & Richards, 1984) and heme (Backes et al., 1986; Light & Olson, 1990). Although heme binding to poly(amino acids) has been reported, either the polymers used have, unlike cytochrome *c*, contained a very high histidine content (Tohjo & Shibata, 1963); the binding has been performed at high pH, as in the

case of heme binding to poly(lysine) (see Tsuchida et al., 1975); or the dissociation constants have been orders of magnitude smaller than those reported here, as in the case of a poly(γ-benzoylglutamic acid) containing a single imidazole [see Tsuchida et al. (1975)]. Ribonuclease, used here as a control, contains four histidines out of 124 amino acids, compared with three histidines out of the 104 amino acids of horse cytochrome c.

Other proteins, including other cytochromes, globins, and proteins with physiological roles in heme complexation, such as hemopexin (Wu & Morgan, 1993) and human serum albumin (Cannistraro, 1983), undergo conformational changes upon binding heme. The noncovalent interaction we observed between heme and cytochrome c is weaker than the affinity of heme for other known heme-binding proteins. Could such relatively weak binding play a role as an initial step in the formation of holocytochrome c? One way of addressing this question is to consider the levels of available heme in cells. It is unlikely that any heme is present in totally unbound form in cells, but pools of readily exchangeable heme have been detected. Such heme is presumed to be only loosely bound to proteins or lipids and to play a role in regulation of heme biosynthesis and other metabolic processes. The concentration of heme in such pools is not definitively established but has been estimated to be on the order of 10^{-7} M in embryonic liver cell from chicken (Granick et al., 1975). This estimate is based on the measured effects of exogenously added heme on cell metabolism and on a hypothetical homeostatic mechanism involving heme oxygenase, which degrades heme with a K_M for heme of about 10^{-6} M, and repression of heme synthesis, which reaches 50% of the maximal effect at 10^{-7} M concentration of added heme. Since the final steps of heme synthesis occur in mitochondria and a pool of newly synthesized loosely bound heme can be detected following pulse-labeling of cells with radioactive heme precursor (Grandchamp et al., 1981), apocytochrome c associated with mitochondria might be exposed to a greater concentration of available heme than that present in other cell compartments. These estimates of available intracellular heme concentrations, when coupled with the measured affinity of heme for apocytochrome c, lead to the prediction that a significant population of heme–apocytochrome c complex exists in cells. The greater apparent specificity of heme binding to apocytochrome c under reducing, as compared to oxidizing, conditions is consistent with reports that heme must be present in the reduced form in order to serve as a substrate for covalent heme attachment to cytochrome c (Nicholson & Neupert, 1989).

Previous studies of cytochrome c folding have used denaturing treatments to unfold holocytochrome c containing heme covalently linked to its two cysteine residues. By analogy to the general effects of disulfide linkages in proteins and because of the extensive hydrophobic surface of heme, such a cross-link might be expected to affect both the kinetics and free energy of folding (Doig & Williams, 1991). Consistent with this, there are indications that some noncovalent heme–protein interactions are preserved in holocytochrome c exposed to denaturing conditions: (1) His 18 appears to be maintained as a ligand of the heme iron even at high concentrations of guanidine hydrochloride (Muthukrishnan & Nall, 1991; Tsong, 1974) or at low pH (Babul & Stellwagen, 1972). (2) The change in exposed surface area of cytochrome c calculated from the change in heat capacity on unfolding is less than would be predicted on the basis of the change in exposed nonpolar groups for a protein of this size (Doig & Williams, 1991). In contrast to previous holocytochrome c-refolding studies, experiments based on the folding of apocytochrome c should be free of preformed heme–protein interactions. The use of noncovalent heme binding to trigger formation of a partially folded state of apocytochrome c may, thus, provide a system that would be useful for the study of the structural details of the folding of apocytochrome c during transport into mitochondria.

The possibility that *in vivo* cytochrome c folding occurs *via* a pathway in which heme is first bound noncovalently to the apoprotein and then the complex is acted upon by heme lyase is currently being tested by studying the role of heme in the initial binding of apocytochrome c to heme lyase and by analyzing effects of mutations in heme biosynthesis on subcellular localization of an altered form of apocytochrome c that is incapable of having heme covalently attached (see Dumont et al., 1991). Covalent heme attachment to cytochrome c occurs in a stereospecific manner. This means that, in order to avoid a situation where 50% of the bound complex can not be converted to holocytochrome c, either formation of the noncovalent complex would have to be stereospecific or a mechanism must exist for flipping the orientation during folding and interaction with the heme lyase.

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