When beef heart apocytochrome c is unfolded, it folds upon noncovalent heme binding (Dumont, M. E., Corin, A. F., and Campbell, G.A. (1994) Biochemistry, 33, 7368–7378). Here, the conformation of the heme-apocytochrome noncovalent complex is compared with that of holocytochrome c. A purification method was designed for obtaining in large amounts apocytochrome c that was shown by amino acid analysis and mass spectroscopy to be chemically intact. The apoprotein and its noncovalent complex were characterized by absorption, fluorescence, circular dichroism, and sedimentation velocity, confirming previous reports. Sedimentation-diffusion equilibrium showed that the apoprotein and its noncovalent complex with heme were monomeric. Surprisingly, whereas apocytochrome c was quite soluble, the noncovalent complex slowly formed heavy aggregates, thus precluding experiments at the concentrations needed for structural studies. Two monoclonal antibodies that bind strongly to distinct antigenic sites on native holocytochrome c were used to probe the noncovalent complex conformation. For both antibodies, the affinity for the noncovalent complex was only about 5–10-fold smaller than that for native holocytochrome c, and about 50–100-fold larger than that for apocytochrome c. These results indicate that the noncovalent complex, although not entirely native, carries some pseudo-native structural motifs.

Investigations on the mechanisms of protein folding have for many years emphasized the role of short range interactions in the formation of secondary structure elements, i.e. α-helices and β-turns/β-sheets, at early stages of the folding process (1–4). Although the necessity of a coupling between secondary structure and some long range interactions, such as specific disulfide bonds, was pointed out long ago (5), the possibility that specific long range interactions may drive the folding from the very beginning of the folding process was not taken seriously until the application to proteins of the notion of “energy landscape” (6). Yet experimental evidence was already available that indicated the need for such long range interactions in promoting or stabilizing secondary structure elements at early stages of the folding of some proteins. Thus, the native secondary structure of hen lysozyme was shown to be recovered in less than 4 ms of folding when the native disulfide bonds were kept intact in the denatured state (7), whereas no detectable secondary structure was present after 4 ms of folding when the denatured protein was reduced (8). Recent studies even showed that the regain of secondary structure during the renaturation/oxidation of reduced lysozyme is very slow and likely to depend strictly on the formation of at least some native disulfide bonds (9).

Similarly, horse cytochrome c folds efficiently only when it carries its cofactor. Indeed, the apoprotein behaves essentially as an unfolded polypeptide chain (10, 11), indicating that the interactions between the polypeptide chain and the heme moiety are needed to achieve a folded state. This was confirmed by the observation that long range interactions between the heme and specific side chains of apocytochrome c are formed during the initial phases of the folding of holocytochrome c (12), suggesting that these interactions contribute to either initiating the folding process or stabilizing very early intermediates, rather than only to the stabilization of the folded state. Moreover, the noncovalent binding of heme to apocytochrome c promotes a structured conformation of the polypeptide chain (13). However, although clearly shown to be definitely folded and compact, this conformation certainly differs from that of native cytochrome c. In particular, the far UV CD and the visible absorption spectra of the noncovalent complex differ from those of the native protein (13). In order to gain some insight to the respective contributions of covalent and noncovalent heme/protein interactions in structuring the polypeptide chain, it seemed to us of interest to find out whether or not the noncovalent complex shares some conformational features with native holocytochrome c. We therefore undertook investigations on the conformation of the noncovalent complex between heme and apocytochrome c.

In the present paper, we describe an easy, large scale purification procedure of apocytochrome c and the characterization by circular dichroism spectroscopy and analytical ultracentrifugation of the resulting apoprotein and of its noncovalent complex with heme. We also report structural investigations of the noncovalent complex, involving measurements by ELISA1 and isothermal titration microcalorimetry of the affinities of two “conformation-specific” monoclonal antibodies for holocytochrome c, apocytochrome c, and the noncovalent heme-apocytochrome c complex.

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¶ To whom correspondence should be addressed. Tel.: 33-1-45688386; Fax: 33-1-40613043; E-mail: goldberg@pasteur.fr.
§ Current address, Unité de Biochimie Structurale, Institut Pasteur, 28 rue du Dr. Roux, 75724 Paris, Cedex 15, France.

1 The abbreviations used are: mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; DTT, dithiothreitol; ITC, isothermal titration calorimetry.
Materials and Methods

Chemicals and Buffers—Heme was obtained as ferrirrophyrin hydroxide (hematin) from Aldrich. Heme stock solutions were prepared in 0.1 M sodium hydroxide, and their concentration was determined spectrophotometrically (εmax = 5.84 × 10⁴ M⁻¹ cm⁻¹). All chemicals used were reagent grade. Unless otherwise stated, the buffer was 50 mM sodium phosphate, pH 7.5, containing 5 mM DTT.

Horse heart holocytochrome c was purchased from Sigma. Holocytochrome c was obtained as described previously (10). 100 mg of holocytochrome c were dissolved in 2 ml of distilled water and supplemented first with 160 mg of silver sulfate dissolved in 18 ml of water and second with 1.6 ml of pure acetic acid. The mixture was incubated at 40 °C for 4 h and centrifuged. The purification of the apocytochrome c thus obtained was achieved as follows. The supernatant (21 ml) was dialyzed overnight at 4 °C against 2 liters of millimolar sodium phosphate, pH 5, and 0.5 mM DTT. The solution was dialyzed at 4 °C against 2 liters of millimolar phosphate, pH 5, 5 mM DTT for 3 h, the buffer was changed and the dialysis carried on overnight. The dialysate was centrifuged as above to remove a fine, yellowish precipitate. The supernatant (29 ml) was separated in 1.5-ml aliquots, lyophilized, and stored at −20 °C in a dessicator with silica gel.

The α subunit of Escherichia coli tryptophan synthase was synthesized as described previously (14).

Monoclonal antibodies 5F8 and 2B5 were obtained, starting from hybridoma cell lines kindly provided by Dr. Barry Nall (Department of Biochemistry, University of Texas Health Center, San Antonio, TX). Hybridoma cells were cultured, injected intraperitoneally into pristane-primed BALB/C mice, and antibodies were purified from the resulting ascitic fluids by ammonium sulfate precipitation of the immunoglobulin lines followed by ion exchange chromatography on DEAE, following previously described protocols (15). The characterization of these antibodies has been reported previously (16). The preparation and characterization of antibody mAb164 was described previously (17).

Protein concentrations were determined spectrophotometrically, using as extinction coefficients those reported for apocytochrome c (10), εmax = 0.92 ml/mg/cm for apocytochrome c (10) and εmax = 1.5 ml/mg/cm for the monoclonal antibodies (18), εmax = 0.46 ml/mg/microgram of tryptophan synthase (19) and εmax = 8.58 ml/mg/microgram for holocytochrome c (20).

Spectroscopic Measurements—Absorption spectra were recorded in a Lambda 2 double beam spectrophotometer (Perkin–Elmer) and fluorescence spectra in a LS-5 double monochromator spectrophuorometer (Perkin–Elmer), both thermostatted at 20 °C. For quantitative heme binding studies, the quenching of the intrinsic fluorescence (excitation at 295 nm, emission at 348 nm) was measured immediately after addition of the heme. The excitation and emission bandwidths were adjusted to 5 nm. When low apocytochrome c concentrations were used (0.4 μM), the solution was supplemented with 100 μg/ml of pure α subunit from Escherichia coli tryptophan synthase in order to prevent adsorption of the apocytochrome onto the walls of the cuvette; because this protein contains no tryptophan, the fluorescence background thus introduced was only moderate. The inner filter and dilution effects on the measurement were corrected for density and viscosity of the solvent to obtain the standard extinction coefficients.

Mass Spectrometry—For molecular weight measurements by mass spectrometry, samples of lyophilized apo or holocytochrome c were dissolved in water:methanol:formic acid (50:50:0.5) and introduced into an API IIIA or a QSTAR Elite (both by Perkin–Elmer) pneumatically assisted electrospray interface. The ionspray probe was held at 4.5 kV, and the orifice voltage was set at 45 V. The mass spectra were monitored continuously from m/z 700–1800 with a scan step of 0.1 and a dwell time per step of 2.0 ms resulting in a scan duration of 22.0 s. Ten scans were averaged for each analysis. Mass calibration of the instrument was accomplished by matching ions of polypropylene glycol to known reference masses stored in the mass calibration table of the instrument. Data were collected on a Power Macintosh 8600/200 and processed through the Biotool software from Serix.

Analytical Ultracentrifugation—Ultracentrifugations were performed in a Beckman Optima-XLA analytical centrifuge, using standard double sector cells with 1.2-mm-thick aluminum centerpieces. The temperature was 20 °C. The cells were scanned at the wavelengths indicated in the text. For sedimentation velocity runs, single scans were performed at constant time intervals. The sedimentation coefficients were analyzed using the XL-AVEL program (Beckman) provided with the analytical centrifuge. The observed sedimentation coefficients were corrected for density and viscosity of the solvent to obtain the standard sedimentation coefficients. For sedimentation-diffusion equilibrium studies, 0.15 μl of sample solution were laid over 50 μl of fluorochrome FC43 (3 M-Minnesota). Radial scans were recorded at 1.5-h intervals for at least 18 h to check that equilibrium was reached. When equilibrium was achieved, 5 or 10 successive scans were averaged to improve the signal to noise ratio in the recordings. The centrifuge was then accelerated to 59,000 rpm to clear the meniscus from any residual protein and determine the absorbance base line. The protein distribution at equilibrium was monitored by a nonlinear regression method was used to extract affinity constant from the saturation curves. Each affinity measurement was done in triplicate.

Isothermal Titration Calorimetry (ITC)—Experiments were performed on the MicroCal MCS ultra-sensitive titration calorimeter (MicroCal Inc., Northampton, MA) using the OBSERVER software provided by the manufacturer for instrument control and data acquisition (23). To improve base line stability, the temperature of the adiabatic jacket was kept 5 °C below the temperature of the experiment by setting the circulating water bath at 10 °C and by activating the jacket feedback power, and temperature was equilibrated for 12 h. During a titration experiment, the protein sample was thermostatted at 20.0 ± 0.1 °C in a stirred (410 rpm) reaction cell (1.3514 ml), and aliquots of the titrant solution were injected from a 100-μl or 250-μl syringe as indicated in the legends of the figures. The reference cell of the calorimeter contained water with 0.05 M sodium acetate. Data points were averaged and stored at 2-s intervals. All buffer solutions were thoroughly degassed by stirring under vacuum before use (buffer compositions are given in the figure legends). Protein samples were prepared in buffer of the same batch to minimize artifacts due to any differences in buffer composition. Titration experiments were performed with protein concentrations in the reaction cell and in the syringe insuring a final titrant/binding-site molar ratio of 2:1 in the reaction cell. Raw calorimeter analyzer after acid hydrolysis of 0.2-mg protein samples in sealed tubes for 20 h at 110 °C in 6 N HCl and 0.2% phenol.

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metric data, i.e. heats released accompanying the addition of aliquots of titrant solution into the protein solution in the reaction cell, were processed using the software package ORIGIN (23, 24). The area under the curve was the total enthalpy of binding, which was calculated using the equation \( \Delta H = \frac{RT}{\Gamma} \ln(K_d) \), where \( R \) is the gas constant, \( T \) the absolute temperature, and \( K_d \) the dissociation constant of the apocytochrome c-heme complex. 

### Results

**Large Scale Purification of Apocytochrome c**—An efficient procedure for cleaving the covalent bonds linking the heme to two cysteine residues in cytochrome c has been previously described (25). Based on this procedure, we devised a new purification method based on the expected difference in solubility between apo- and holocytochrome c. Indeed, in the holo-protein a large number of hydrophobic residues are buried in the core of the molecule, in contact with the heme (26), whereas in the unfolded apoprotein, these residues are exposed to the solvent. Thus, the heme and apoprotein were first resolved by treatment with silver sulfate (10) and, after centrifugation and dialysis (see under “Materials and Methods”), the resulting mixture was treated with 1% ammonium sulfate. This was enough to completely precipitate apocytochrome c, whereas holocytochrome c remained entirely soluble under these conditions. After centrifugation, the ammonium sulfate precipitate was dissolved in 6 M guanidinium chloride and 50 mM ammonium acetate at pH 5 containing 0.5 mM DTT. The guanidinium was then removed by extensive dialysis against 50 mM ammonium acetate, pH 5, supplemented with 5 mM DTT. After dialysis, the apocytochrome c was obtained in a soluble form. A typical purification based on these steps is described in Table I. 

The absorption spectrum of the resulting protein showed very little residual absorbance in the 400 nm region (Table I), indicating that no significant amount of heme remained in the purified apocytochrome c. However, a very small shoulder near 350 nm could still be observed in some preparations (\( A_{280}/A_{250} < 0.04 \)). No attempt was made to identify the nature of this trace contaminant.

**Physicochemical Properties of the Apo-enzyme**—Purified apocytochrome c was submitted to amino acid analysis to verify its chemical integrity. The amino acid composition expected from the known sequence of cytochrome c was obtained. The two cysteinyl side chains were already reported to be obtained in the reduced state (11). The absorption and fluorescence spectra showed that the tryptophan residue was present and intact in the apocytochrome c preparation. The mass spectrum of the apo-cytochrome c preparation showed a predominantly peak at an estimated mass of 11,743.0 ± 0.7 Da. The excess of 42 Da over the mass calculated from the known amino acid sequence of horse cytochrome c (11701.6 Da) corresponds exactly to an acetylation, a chemical modification known to exist at the N-terminal extremity of cytochrome c. The same excess in mass was observed with holocytochrome c, indicating that the acetylation indeed existed in the starting material. These observations lead to the conclusion that the methionine residues have not been altered by the procedure used to prepare apocytochrome c.

The far UV CD and the fluorescence emission spectra of apocytochrome c were recorded (data not shown) and found to be essentially identical to those previously reported (10, 11). The far UV CD spectrum showed no signal characteristic of secondary structure, and the fluorescence emission spectrum was that of solvent exposed tryptophan residues, two features confirming that apocytochrome c appears devoid of detectable organized secondary and tertiary structure.

The sedimentation coefficient of apocytochrome c (30 μM) in 50 mM potassium phosphate, pH 7.5, and 5 mM DTT was determined by analytical centrifugation at 20 °C and 59,000 rpm. The observed sedimentation coefficient was 1.08 ± 0.05 S. After correction for the density and viscosity of the solvent, the standard sedimentation coefficient was found to be \( s_{20,w} = 1.13 ± 0.05 \) S. From this value and from the molecular weight of N-acetylated apocytochrome c (11,744 Da), one could estimate the Stokes radius of the apoprotein to be about 25 Å, a value compatible with that determined by dynamic light scattering under similar solvent conditions (27). The sedimentation profile showed a unique, symmetrical boundary, suggesting that the preparation of apocytochrome c contained no low molecular weight aggregates. This was confirmed by observing that the absorptions at 280 nm of a 62 μM solution of apocytochrome c in the centrifugation cell immediately after reaching 5000 and 59,000 rpm were, within the experimental precision (i.e. ± 2%), identical. Moreover, these plateau absorbances were equal to the absorbance measured in a spectrophotometer before the centrifugation, indicating the absence heavy aggregates.

**Physicochemical Properties of the Noncovalent Complex**—Apo-cytochrome c was saturated with heme as described previously (13), and its spectral properties were investigated under reducing conditions (5 mM DTT) in the absence of cyanide. As previously reported, the absorption spectrum of the heme-apocytochrome c mixture showed a Soret absorption band at around 406 nm that, after a rapid initial change, underwent a slow further increase. The fluorescence spectrum of the mixture showed a strong quenching of the tryptophan fluorescence emission, which was used to monitor the formation of the heme-cytochrome c complex. The results (not shown) from 5 experiments conducted at apocytochrome c concentrations of 0.4 and 2.1 μM showed simple binding with a dissociation constant of 1.4 ± 0.2 μM and 2 hemes bound per polypeptide chain, in excellent agreement with previous reports (13). The far UV CD spectrum of the noncovalent complex showed an increase in the amplitude of the ellipticity in the 220 nm region. This confirmed previous reports indicating that the far UV CD spectrum of the noncovalent complex, although quite different from that of native holocytochrome c, reflects the presence of some organized secondary structure.

The hydrodynamic properties of the noncovalent complex were then investigated. A solution of apocytochrome c (4 or 8 μM) was partly saturated with a limiting amount of heme (total concentration, 2 or 4 μM, respectively) to minimize possible “nonspecific” heme binding and optical problems due to the very poor solubility of free heme at neutral pH. The sedimentation of the noncovalent complex was specifically observed by scanning the centrifugation cell at 420 nm, where apocyto-
Apocytochrome c has no absorption. The standard sedimentation coefficient thus obtained was $s_{20,w} = 1.88 \pm 0.06$ S, a value identical to that determined for native holocytochrome c ($s_{20,w} = 1.80 \pm 0.04$ S). Assuming that the noncovalent complex is monomeric, this indicates that the noncovalent complex is as tightly packed as native holocytochrome c, with a Stokes radius estimated to be about 16.2 Å.

That the noncovalent complex is monomeric was verified by sedimentation-diffusion equilibrium of the noncovalent complex. A 6.2 μM solution of apocytochrome c was saturated with 6.2 μM heme and submitted to centrifugation at 40,000 rpm and 20 °C. The centrifugation cell was scanned for absorbance at 560 nm, a wavelength at which only the heme-cytochrome c complex absorbed, ensuring that neither unsaturated apocytochrome c nor free heme that might exist in the solution would contribute to the absorption. The absorbance distribution after 18 h of centrifugation was analyzed. The experimental data were fit using as a model an ideal solution with a unique molecular species. The best fit provided an estimate of 12,830 Da for the molecular weight of the heme-apocytochrome c complex, a value reasonably close to those calculated for the monomeric noncovalent complex between the apoprotein and one or two hemes (12,360 and 12,976 Da, respectively). However, whereas equilibrium seemed at first to have been reached after 18 h, the protein pattern went on evolving slowly, with a progressive decrease in the total protein concentration. This suggested that the protein might undergo a slow aggregation during the centrifugation.

Slow aggregation was confirmed as follows. Apocytochrome c and heme (6.2 μM each) were mixed and incubated for 3 h, at which time the absorption spectrum was stable. The solution was then introduced in the centrifugation cell, accelerated first to 5,000 rpm and then to 59,000 rpm. The absorbance at 560 nm was recorded immediately after reaching 5000 and 59,000 rpm. The values observed were 0.785 and 0.735, respectively, indicating that about 7% of the noncovalent complex were pelleted during the acceleration at high speed. This result confirmed that unlike apo- and holocytochrome c, which are stable in solution, the noncovalent complex is prone to aggregate and must therefore be prepared extemporaneously before use.

Finally, the CD spectrum of the noncovalent complex was also investigated in the absorption region of the heme and compared with that of holocytochrome c. In a control experiment with free heme at the same concentration, no CD signal was detected, indicating that the spectrum observed with the noncovalent complex indeed corresponded to heme bound to apocytochrome c. Comparison of the two spectra in Fig. 1 clearly indicated that the environment of the heme in the two species were largely different.

From all of these studies, it appeared that except for its tight packing and for its far UV CD spectrum suggesting the presence of some organized secondary structure, the noncovalent complex showed no direct evidence of sharing structural features with native holocytochrome c. Hence, the need to analyze in more details the conformation of the noncovalent complex.

Neither x-ray crystallography nor NMR could be envisaged for these studies because of the propensity of the complex to aggregate, a feature that precluded the use of the high protein concentrations needed for such experiments. We therefore undertook a conformational study of the noncovalent complex, using two monoclonal antibodies that had been reported to bind selectively to native holocytochrome c.

**Immunochemical Properties of Apocytochrome c, Holocytochrome c, and the Noncovalent Complex—**Two monoclonal antibodies, 5F8 and 2B5, that bind strongly to native holocytochrome c have been described (16). Their epitopes lie on opposite sides of the native antigen. Antibody 5F8 recognizes a region comprising lysine 60, near the N-terminal end of an α-helix. Antibody 2B5 recognizes residues around proline 44 in a type II β-turn (16). These two antibodies appeared unable to bind apocytochrome c, and hence as specific of native holocytochrome c (28). They were therefore chosen to find out whether or not the “folded” noncovalent heme-apocytochrome c complex shares native-like epitopes with holocytochrome c.

The affinities of both antibodies for native holocytochrome c and for the noncovalent heme-apocytochrome c complex were first determined, using the competition ELISA method (22) under conditions where the antibody immobilized during the ELISA represented less than 10% of the free antibody (22) and where saturation of the antibody with the antigen was sufficiently high for straightforward interpretations of the data (29). The results of such measurements are depicted in Fig. 2. Fitting binding isotherms to the data provided the affinities reported in Table II, which indicated that the two antibodies recognized the noncovalent complex with affinities that differed by a factor of about 10 only from their affinities for native holocytochrome c. The reduced apparent affinity of the antibodies for the noncovalent complex as compared with holocytochrome c could be interpreted in two ways. Either only 10% of the cytochrome c molecules were recognized by the antibodies with the same affinity as native cytochrome c, or all molecules were recognized, but with a reduced affinity. To sort this alternative, a solution containing 4 μM apocytochrome c, 2 μM heme (so that no significant amount of free heme would remain in solution), and 6 μM 5F8 antibody (i.e. 12 μM antigen binding sites) was submitted to analytical centrifugation at 58,000 rpm and 20 °C, and the cell was scanned at 420 nm at 10-min intervals. The sedimentation profile showed a unique symmetrical boundary ($s_{20,w} = 7.3$ S). No slowly sedimenting material could be detected at 420 nm. This demonstrated that all the heme-saturated apocytochrome c molecules were bound to the antibody. In a control experiment with a nonspecific monoclonal antibody, all of the hemoeprotein migrated as the free noncovalent complex, indicating that the binding observed with mAb5F8 was indeed specific. This indicated that all, rather than only a small fraction, of the heme-saturated molecules were recognized by the specific antibodies.

The two epitopes thus appeared to be present in a nearly native conformation in the noncovalent complex. This interpretation relied on the assumption that the monoclonal antibodies could not recognize the unfolded cytochrome c molecule. It would be interesting to determine whether recognition of both epitopes is indeed necessary for the formation of the native conformation of the noncovalent complex. Such an apparent conformational restriction could be the result of a steric hindrance that would not be possible in the case of the unfolded protein. This possibility is currently under investigation.
seemed important to check the validity of this assumption by
determining the affinities of both antibodies for apocytochrome
c. Because apocytochrome c has a strong tendency to stick on
the ELISA plates, a prohibitively high background of binding of
the free antibody to this "over-adsorbed" antigen precluded the
use of the ELISA competition test at the high antigen concen-
trations needed to measure low affinities. None of our attempts
to reduce this nonspecific adsorption was successful. Similarly,
the BIACORE method could not be used because of a strong
adsorption of apocytochrome c onto the chips. We therefore
turned to isothermal titration microcalorimetry, a method al-
ready used previously (30) to study the binding of mAbs 2B5
and 5F8 to holocytochrome c.

In a first set of experiments, mAb5F8 (10 µM) was placed in
the calorimeter cell, and apocytochrome c (250 µM) was injected
from the syringe in 10 µL aliquots. The diagram obtained (Fig.
3A) was analyzed assuming a unique set of independent bind-
ing sites. The best fit to the experimental data was obtained
with an affinity of 8.7 × 10^5 ± 0.7 × 10^5 M^{-1} and 2.02 ± 0.03
binding sites per IgG molecule. In a symmetrical experiment,
apocytochrome c (4 µM) was placed in the calorimeter cell and
mAb5F8 (18.5 µM) in the syringe. Using a unique set of inde-
pendent binding sites as a model, the best fit was obtained with
an affinity of 3.0 × 10^6 M^{-1} and 2.2 binding sites per IgG
molecule. The difference by a factor of 3 in the affinities ob-
tained under the two sets of experimental conditions may be
explained by the large difference in the apocytochrome c con-
centration (250 and 4 µM for the antigen in the cell or in the
syringe, respectively). Whereas apocytochrome c was mono-
meric in analytical centrifugation experiments performed at
about 4 µM (see above), significant aggregation might occur at
250 µM. This assumption was supported by the observation
that when kept at high concentration for several hours, apocy-
tochrome c showed complex titration patterns that could be
best explained by the dissociation of aggregated material (data
not shown). For this reason, only experiments in which apocy-
tochrome c solutions were prepared extemporaneously were
taken into consideration. In a control experiment with the
nonspecific antibody mAb164 (directed against tryptophan syn-
phase), no specific enthalpy change could be detected upon
mixing with apocytochrome c (data not shown). It thus could be
concluded that the apparent affinity of mAb5F8 for apocyto-
tochrome c was between 8.7 × 10^5 and 3 × 10^6 M^{-1}, i.e. about
1000–3000-fold smaller than that (the mean of the ELISA and
microcalorimetric determinations) of mAb5F8 for native holoc-
tyochrome c. To find out whether this "residual" affinity of the
antibody for apocytochrome c was due to a contamination of the
antigen preparation with 0.1% of holoprotein or to a weak

**FIG. 2. Determination of the affinity of mAb2B5 for the noncovalent apocytochrome c-heme complex by competition ELISA.** Aliquots of a mAb2B5 solution at a fixed concentration were mixed with equal volumes of a stoichio-
metric mixture of apocytochrome c and heme at various concentrations. After a
2-h incubation at room temperature (20 ± 2 °C), the amount of unsaturated anti-
body in the solution was measured by ELISA as indicated under "Materials and
Methods." The results were plotted in the Klotz representation as suggested by
Friguet et al. (23) and fit by linear regres-
sion. The slope of the straight line thus obtained equals the dissociation constant
(i.e. reciprocal of the affinity).

**TABLE II**

| Antibody ⇒ antigen | 2B5, K_{max} | 5F8, K_{max} | 2B5 | 5F8 |
|--------------------|--------------|--------------|-----|-----|
|                    | m^{-1}       | m^{-1}       | ΔG° | ΔH° |
| Holocytochrome     |              |              |     |     |
| ELISA              | 5 × 10^9 ± 1.1 × 10^9 | 3 × 10^9 ± 9 × 10^8 | H | F |
| Microcalorimetry   | 2 × 10^9 ± 3 × 10^6 | 1.8 × 10^9 ± 2.5 × 10^6 | H | F |
|                    |              |              | ±11.1 | ±17.5 | ±21.7 | ±12.7 | ±22.2 | ±33.1 |
|                    | ±0.1 | ±0.2 | ±0.8 | ±0.1 | ±0.2 | ±0.5 |
| Apocytochrome + heme |            |              |     |     |
| ELISA              | 7 × 10^7 ± 1.2 × 10^7 | 3 × 10^7 ± 9 × 10^7 | H | F |
| Microcalorimetry   | 1.3 × 10^6 ± 4 × 10^5 | 8.7 × 10^5 ± 7 × 10^6 | H | F |
|                    | 1.6 × 10^4 ± 8 × 10^4 | 3.0 × 10^4 ± 6 × 10^4 | H | F |
|                    | ±8.5 | ±13.4 | ±16.7 | ±8.02 | ±15.4 | ±24.4 |
|                    | ±0.2 | ±0.3 | ±1.6 | ±0.05 | ±0.3 | ±1.2 |
|                    | 3.4 × 10^7 ± 1.5 × 10^8 (15%) | 2.9 × 10^6 ± 6 × 10^6 (85%) | H | F |

* In kcal/mol.
* In cal/mol/K.
* Only one binding site per IgG molecule was obtained.
* Two binding sites with the affinity indicated were obtained per IgG molecule when assuming identical noninteracting binding sites.
* Two binding sites with the affinities indicated were obtained per IgG molecule when assuming identical but interacting binding sites.
* Inverted titration": the antigen was in the ITC cell, and the antibody was added from the syringe.
binding of the antibody to heme-free apocytochrome c, apocytochrome c (250 μM) was supplemented with 1.25 μM mAb5F8 (i.e. 2.5 μM binding sites), an amount sufficient to presaturate up to 1% of holoprotein that might be present in the apoprotein solution. This mixture was placed in the syringe and injected in the cell containing mAb5F8 (10 μM). The affinity obtained by fitting, 8.7 × 10^5 M⁻¹, was indistinguishable from that obtained in the experiment of Fig. 3, thus ruling out that the residual affinity of mAb5F8 for apocytochrome c might be caused by an immunoreactive contaminant.

Experiments similar to that described in Fig. 3A were repeated with mAb2B5. The results obtained revealed a more complex binding reaction, with two distinct binding modes (Fig. 3B). Using as model a unique set of independent binding sites provided a rather satisfactory fit, with an affinity of about 2 × 10^8 M⁻¹ and only one binding site per antibody molecule, with the first, sharp titration mode, but failed to describe the second part of the titration curve. A good fit of the complete titration curve could be obtained using a model with two identical noninteracting binding sites per antibody molecule (data not shown). That only about one site per mAb molecule could be saturated with high affinity confirmed that the binding of the antigen to mAb2B5 is anticooperative. Contrary to mAb2B5, mAb5F8 showed no anticooperativity. This was demonstrated in a titration experiment specially designed to ensure precise measurements of a high affinity constant (Fig. 4). A mixture of mAb5F8 (1.64 μM) and holocytochrome c (2.8 μM) was introduced in the calorimeter cell in order to start the titration just before the saturation transition, and holocytochrome c (25.3 μM) was injected from the syringe in small aliquots (3 μl) to ensure the collection of a sufficient number of significant data points. The curvature of the diagram in Fig. 4 indicated that the experiment should permit resolution of the binding constant from the titration data. These data could be well described according to a model with two identical noninteracting binding sites per antibody molecule. The best fit was obtained for 1.99 sites per mAb5F8 molecule, with an affinity of 1.8 × 10^9 ± 0.25 × 10^9 M⁻¹. Thus, mAb5F8 showed neither cooperativity nor anticooperativity for antigen binding, confirming the results obtained with apocytochrome c.

Conformational Changes of Apocytochrome c upon mAb Binding—The possibility that a conformational change of apocytochrome c might accompany its binding to the monoclonal antibodies was examined by measuring the affinity of one of the mAbs for the preformed complex between the apoprotein and the second mAb. It was first verified that as expected from the positions of the corresponding epitopes on native cytochrome c, the two mAbs could bind simultaneously to the antigen. For
that purpose, holocytochrome $c$ (4 $\mu$M) was first mixed with mAb2B5 (5 $\mu$M) and incubated for 15 min at 20 °C. The mixture was supplemented with mAb5F8 (5 $\mu$M), incubated for 1 h at 20 °C, and submitted to analytical centrifugation. The rotor was accelerated to 55,000 rpm, and scans at 410 nm were made at 15-min intervals. The absorbances in the cell after reaching full speed was the same as that determined for the solution before centrifugation, indicating that no large aggregates had been formed. The sedimentation of the light absorbing material (i.e. cytochrome $c$) showed a unique, symmetrical boundary, with an observed sedimentation of 10.6 S. The value observed in a control experiment with only holocytochrome $c$ and mAb2B5 was 7.2 S. Comparing these two values shows that holocytochrome $c$ was bound to the two antibodies simultaneously. Binding of mAb5F8 to the preformed complex between apocytochrome $c$ and mAb2B5 was therefore investigated by microcalorimetry. A mixture of apocytochrome $c$ (4 $\mu$M) with mAb2B5 (5 $\mu$M) was titrated by injecting 8-$\mu$l aliquots of mAb5F8 (18.5 $\mu$M) from the syringe. The results, represented in Fig. 5A (filled symbols), showed a bimodal titration curve. A control experiment in which apocytochrome $c$ alone was titrated with mAb5F8 showed only the second binding mode (Fig. 5A, open symbols). This demonstrated that the first binding mode observed in the presence of mAb2B5 reflected the presence of a population of apocytochrome $c$-mAb2B5 complex with high affinity for mAb5F8. Attempts to fit the data according to models in which the antigen was homogeneous were unsuccessful, regardless of the assumptions made on the affinities, number of binding sites per mAb5F8, and possible interactions between sites. Therefore, the two binding modes observed in Fig. 5A were further characterized experimentally, by varying the concentration of the mAb5F8 solution injected. The first binding mode (corresponding to the higher affinity) was characterized using a low mAb5F8 concentration (2 $\mu$M). The results, reported in Fig. 5B, were analyzed using a model with one set of independent binding sites as described above, but with one modification in the fitting procedure: whereas the concentration of reactive antigen (i.e. the cytochrome c-mAb2B5 complex) was let free to vary, the stoichiometry of the binding reaction was maintained constant and equal to two antigen binding sites per mAb5F8, the stoichiometry determined from the results in Fig. 5A. As shown in Fig. 5B, a meaningful fit of the first transition was obtained for an antigen concentration of 0.6 $\mu$m (i.e. 15% of the total apocytochrome $c$ concentration), giving an association constant of $3.4 \times 10^7 \pm 0.4 \times 10^7$ $\text{M}^{-1}$. The second binding mode (corresponding to the lower affinity) was characterized using a high mAb5F8 concentration (28 $\mu$M). The results presented in Fig. 5C confirmed those of Fig. 5A. Fitting the second binding transition with a model with one set of independent binding sites provided values of $2.1 \pm 0.07$ for the number of binding sites per mAb5F8 and $2.9 \times 10^6 \pm 0.6 \times 10^6 \text{M}^{-1}$ for the affinity. These values were indistinguishable from those obtained in the control without mAb2B5 (2.2 $\pm$ 0.1 and 3.0 $\times$ 10$^6$ $\pm$ 0.6 $\times$ 10$^6$ $\text{M}^{-1}$, respectively). Thus, the population of apocytochrome $c$-mAb2B5 complex molecules was shown to contain about 15% of molecules recognized by mAb5F8 with a 10–15-fold higher affinity than the remaining 85% of the molecules, which were recognized with the same affinity as free apocytochrome $c$.

The number of binding sites per mAb and affinities obtained in all the experiments described above are summarized in Table II, together with the free energy (calculated from the equilibrium constant), enthalpy (obtained from the microcalorimetry measurements), and entropy (calculated from the free energy and the enthalpy) of binding.
DISCUSSION

As compared with previous reports, the present work has brought new results on the purification, immunoreactivity, and conformation of apocytochrome c and its noncovalent complex with heme. These various aspects will be discussed successively.

By introducing a new method (differential ammonium sulfate precipitation) to separate the apoprotein from residual heme-containing molecules, a protocol has been set up for the large scale preparation of apocytochrome c. Hundreds of milligrams of pure apocytochrome c can thus easily be obtained in one run. It has been demonstrated that this procedure does not affect the integrity of the polypeptide chain. In particular, our mass spectrometry measurements demonstrate that the methionines are not oxidized, which is of particular importance because, in native cytochrome c, the sulfur of methionine 80 is engaged in a coordination bond with the iron of the heme (26).

All of the spectral and hydrodynamic properties that we observed with our preparation of apocytochrome c fully confirmed those previously reported for the free apoprotein (10, 11) and the noncovalent complex (13) obtained with previous purification procedures. We also showed that despite the similarities of the absorption spectra of the heme in holocytochrome c and in the noncovalent complex (13), the heme environment appears drastically different in these two species, because their CD spectra in the visible range are widely different. Additionally, we brought direct evidence indicating that the noncovalent complex is monomeric but shows a strong tendency to slowly form high molecular weight aggregates even in dilute (6 μM) solution. Surprisingly in view of its very low solubility in the presence of ammonium sulfate, apocytochrome c is much less prone to aggregation, even though we observed during microcalorimetry measurements that it also forms some aggregates upon standing for several hours in concentrated (250 μM) solutions.

The results obtained from antibody binding studies will now be discussed. From a methodological point of view, it is worth emphasizing the complementarity of the ELISA- and ITC-based methods for measuring affinities. Indeed, low affinities such as those of the two antibodies for apocytochrome c could well be measured by ITC but not by ELISA. Conversely, very high affinities, such as those for native holocytochrome c, were easily determined by ELISA but required a modification of the standard ITC procedure. One should, however, point out that in contrast to the limitations previously encountered (31), the increased sensitivity of presently available microcalorimeters provided reasonable titration calorimetry estimates of the affinities of mAbs 2B5 and 5F8 for holocytochrome c. Despite the fact that due to these high binding constants, the ITC affinity measurements were made close to the limit of sensitivity of the method, the values obtained by ELISA and ITC were in reasonably good agreement. The agreement was particularly good for the mAb5F8/holocytochrome c complex, although its dissociation constant is as low as 3 \times 10^{-10} M, when the titration was “sensitized” by premixing the antigen with the appropriate amount of antibody (see under “Results”). The values we obtained were, however, significantly smaller than those previously reported for mAb5F8 (32) and mAb2B5 (30). The latter were measured at 25 °C, a temperature 5 °C above that of the present experiments (20 °C, Table II). Prior to comparison, values of the affinity constants must therefore be corrected for the temperature difference. Based on the changes in heat capacities (ΔCp) accompanying the two binding reactions measured by titration calorimetry by Raman et al. (30), corrected values of the affinity constants at 20 °C were of 2.3 \times 10^{10} M^{-1} and 2.6 \times 10^{9} M^{-1} for mAb5F8 and mAb2B5, respectively. Hence, the affinity constants of the two mAbs determined in this study (Table II) were 13-fold smaller than those derived at the same temperature from the studies of Raman et al. (30, 31).

The reason why the affinity of mAb5F8 for cytochrome c had been overestimated in previous studies has already been discussed (32). The discrepancy between our values and that reported previously for mAb2B5 is more difficult to explain. It may originate from the fact, demonstrated in our microcalorimetry measurements, that this antibody binds efficiently only one cytochrome c molecule per IgG, a feature that the authors did not take into account in their fitting model (30).

The microcalorimetry experiments that we report provided dissociation constants in the micromolar range (Table II) for the complexes between the two antibodies and apocytochrome c, whereas this method failed to detect any interaction between this antigen and a control antibody (mAb164) directed against a foreign protein. Moreover, premixing apocytochrome c in a 100:1 molar ratio with mAb5F8 ruled out the possibility that the enthalpy of binding detected by microcalorimetry might be due to a small contamination of the apoprotein preparation by residual holocytochrome c. Thus, contrarily to earlier conclusions, antibodies 2B5 and 5F8 recognize the apoprotein. Although their affinities for this antigen are much smaller than for holocytochrome c (by factors 270 and 2700 for 2B5 and 5F8, respectively), these weak but significant interactions should be taken into account whenever these antibodies are used at high concentrations. Thus, in experiments aimed at monitoring the regain of native-like epitopes during the refolding of holocytochrome c, antibody binding sites concentrations ranging between 13.3 and 48 μM were used (28). These concentrations were at least 10-fold higher than the dissociation constants that we determined for the corresponding antibodies. Thus, under these conditions, the antibodies could practically saturate apocytochrome c, i.e., the unfolded protein. This indicates that the antibodies might bind to the polypeptide chain even before it folds into a native-like conformation. This casts some doubts on the conclusion that the early holocytochrome c folding intermediates formed within less than 100 ms contain native-like epitopes (28).

The large difference in the affinities of mAbs 2B5 and 5F8 for apo- and holocytochrome c confirm that these antibodies can indeed be used to probe the conformation of the polypeptide chain. Hence, because both antibodies recognize the noncovalent complex much better than unfolded apocytochrome c and nearly as well as native holocytochrome c, one can conclude that the noncovalent heme-apocytochrome c complex carries pseudo-native epitopes. What does “pseudo-native” mean? Two extreme points of view could be adopted. One is that in the absence of antibody, each individual molecule of the noncovalent complex would carry a slightly distorted epitope that can be recognized by the antibody. The ratio of about 10 between the affinities of the native and the distorted epitope could then result from the loss of as little as one elementary interaction with a free energy of about 1.5 kcal/mol, like a hydrogen bond or one hydrophobic contact. Alternatively, it could reflect an “induced fit” of the epitope upon binding to the antibody, which would cost about 1.5 kcal/mol and therefore decrease the affinity by a factor 10. Another point of view, based on the two-state hypothesis for protein folding and adopted by Furie et al. (33) in their “K\_fold” model, would be that the antibody recognizes only the native antigen and that the affinity exhibited for a distorted antigen reflects a pre-existing equilibrium between the unfolded, nonimmunoreactive conformation of the protein and its native, fully immunoreactive conformation. Accordingly, the noncovalent complex would exist in a dynamic equilibrium between the unfolded and native conformations of cytochrome...
c. The ratio of the apparent affinity of a monoclonal antibody for the noncovalent complex (K_{nc}) to the real affinity of that antibody for holocytochrome c (K_{h}) would then be related to the equilibrium constant (K_{conf}) between the nonreactive and the native conformations of the protein in the population of noncovalent complex molecules by the equation K_{conf} = K_{nc}/K_{h} - K_{nc} (33). Because the folding of a small protein such as cytochrome c is highly cooperative, a strong prediction of this model is that the value of K_{conf} should be independent of the antibody used, because it would reflect the intrinsic conformational equilibrium of the antigen. Within the precision of the experimental measurements, this prediction was reasonably well verified for the noncovalent complex. Indeed, the value of K_{conf} was estimated (from the average of the ELISA and microcalorimetric determinations of K_{h}) to be 0.25 for mAb2B5 and 0.14 for mAb5F8. Thus, the immunoreactivity of the noncovalent complex seemed at first sight compatible with the existence of a cooperative conformational equilibrium between the unfolded and the native conformations of the polypeptide chain.

The K_{conf} model assumes that the antibody has no affinity at all for the unfolded antigen. Yet the two antibodies show a small, but certainly not negligible, affinity for apocytochrome c. Again, one might assume that these small affinities in fact reflect the existence of a spontaneous equilibrium between a small fraction of native-like molecules and a majority of unfolded, nonimmunoreactive molecules in the apocytochrome c. Accordingly, comparing the affinities of both antibodies for apocytochrome c indicates that the K_{conf} would be about 4 \times 10^{-3} for mAb2B5 and 3.5 \times 10^{-4} for mAb5F8. Then, about 0.1% only of the molecules would be native, a fraction that by no means could be detected by the spectroscopic or hydrodynamic methods used to characterize apocytochrome c. That the values of K_{conf} of apocytochrome c estimated for the two antibodies differ by a factor about 10 suggests that a concerted conformational equilibrium between unfolded and native apocytochrome c cannot account for the small immunoreactivity detected in the apoprotein with the two antibodies. This conclusion is supported by the comparison of the enthalpies of binding of both antibodies to apo- and holocytochrome c. Indeed, the K_{conf} model predicts that the difference between the enthalpies of binding of the “distorted” antigen and of the native antigen to the antibody should be equal to the enthalpy of the transition from the unfolded to the native conformation. Hence, the enthalpy differences determined for both antibodies should be similar. The same should hold for the entropy differences. Whereas the precision of the free energies (and hence of the entropies) of binding is not very good because they rely on the determination of the equilibrium constants, the values of the enthalpies of binding can be determined much more precisely from the total heat exchange during the transition. The values of \Delta H (i.e. \Delta H_{apo} - \Delta H_{holo}) calculated for mAbs 2B5 and 5F8 from the enthalpies of binding reported in Table II are 4.1 and 7.2 kcal/mol, respectively. The large difference between these two values is clearly not compatible with the prediction made on the basis of a concerted conformational equilibrium between fully unfolded and native apocytochrome c molecules. The basic assumption of the K_{conf} model is therefore definitely not acceptable in the case of apocytochrome c. Rather, it seems likely that the two epitopes undergo nonconcerted equilibria or induced fits.

Yet the conformations of the two epitopes cannot be considered as entirely independent within the apocytochrome c polypeptide chain. Indeed, the binding of mAb2B5 to its epitope on apocytochrome c results in an increased affinity of mAb5F8 for a fraction (about 15%) of the antigen molecules. We shall first discuss the properties of the 2B5/apocytochrome c molecules that bind mAb5F8 with a high affinity. This affinity is about 10-fold higher than that for apocytochrome c alone, but still 100-fold lower than that for holocytochrome c. Thus, the conformation of the polypeptide chain is certainly not entirely native. Yet the binding of mAb2B5 appears to have either shifted a preexisting conformational equilibrium, or induced a conformational change, that brings the polypeptide chain somehow closer to its native conformation and creates a better fit between mAb5F8 and its cognate epitope. The amplitude of the enthalpy of binding of mAb5F8 to these molecules is unusually large (−118.2 kcal/mol) when compared with the binding enthalpies of monoclonal antibodies to protein antigens. In particular, it is about 5-fold larger than its binding enthalpy to native holocytochrome c. At the same time, the amplitude of the binding entropy is also exceptionally high (−368 cal/mol/K) and corresponds to about 11-fold the entropy of binding of mAb5F8 to native holocytochrome c. This strongly suggests that the binding of mAb5F8 to the “high affinity” mAb2B5/apocytochrome c molecules is accompanied by an important conformational change of the polypeptide chain. Considering that the measured enthalpy (or measured entropy) corresponds to the sum of the enthalpy (respectively entropy) of this conformational change and of the enthalpy (respectively entropy) of binding of mAb5F8 to the reorganized molecule, one can estimate to about 96 kcal/mol and 335 cal/mol/K the enthalpy and entropy, respectively, associated with the change in conformation undergone by the mAb2B5-bound apocytochrome c when it binds to mAb5F8. This suggests that within the 2B5/apocytochrome/5F8 ternary complex, apocytochrome c becomes highly ordered, as shown in particular by the strongly negative value of the entropy. Although no direct structural evidence exists that supports this hypothesis, one may speculate that this ordered structure may be close to the native conformation of holocytochrome c. Regardless of the exact nature of this conformation, these results indicate that like heme binding, the simultaneous binding of the two monoclonal antibodies to apocytochrome c represents a case where ligand binding is the principal determinant of a protein conformation, as nicely illustrated in the case of the p21H ras protein (34) and extensively discussed for a variety of proteins (35).

Finally, the fact that only a fraction of the mAb2B5-bound apocytochrome c molecules have a high affinity for mAb5F8 suggests that the binding of mAb2B5 to its epitope hinders some intramolecular movements within the apocytochrome c polypeptide chain, thus preventing the interconversion of two subpopulations of mAb2B5/apocytochrome c complexes, one with high affinity and one with low affinity for the second antibody mAb5F8. As discussed above, the binding of mAb2B5 may represent a key interaction in the antibody induced folding of apocytochrome c. One may therefore draw an analogy between the low affinity subpopulation of mAb2B5/apocytochrome c molecules and kinetically trapped folding intermediates that need to overcome the activation barrier surrounding a metastable state before they can proceed to the native conformation.

In conclusion, we wish to point out the wealth of information that conformation-specific monoclonal antibodies can provide in a case where powerful structural approaches such as NMR or x-ray crystallography are inapplicable to investigate protein conformation and dynamics. Not only did they provide valuable information on the noncovalent heme-apocytochrome c complex, but they also shed some light over the folding properties of the cytochrome c polypeptide chain.

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