Probing the Conformation and Orientation of Adsorbed Protein Using Monoclonal Antibodies: Cytochrome c_3 Films on a Mercury Electrode

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Abstract: A novel use of monoclonal antibodies to probe adsorbed protein conformation is described. Previous electrochemical studies (Zhang, D. B. et al. *Anal. Chem.* **1994**, *66*, 3873−3881) described the characteristics of the potential-dependent adsorption of *Desulfovibrio vulgaris* (Hildenborough) cytochrome c_3 on a mercury electrode. Monoclonal antibodies were generated with epitopes in the vicinity of heme 1. These antibodies were utilized to confirm the existence of three conformationally distinct electrochemical forms depending upon the applied potentials of open circuit (∼ −0.05 V), −0.7 V, and −1.2 V vs AgCl/Ag reference. In all three conformations, the cytochrome c_3 was in a denatured state when compared to soluble protein. When the charge on the electrode was changed from positive to negative (open circuit to −1.2 V), heme 1 remained oriented toward the solution even though the heme 1 region possesses a high positive charge.

Introduction

Protein structure and function are very important for studies using enzymes, for sensors, and for many bioanalytical applications in which the sensitivity of the assay may depend on the orientation and conformation of the molecule that generates the response. Frequently, such applications use molecules that are adsorbed on surfaces. It is important to know the orientation and conformational changes that the molecules undergo during the adsorption phase. Current methods for protein conformation determination (NMR, CD, etc.) are best applied to protein solutions.[1,2] These methods are not generally very useful for resolving the conformational and orientational characteristics of an adsorbed protein at the very low concentrations typically resulting from surface adsorption. NMR can resolve the structural characteristics of adsorbed peptides attached at high concentrations to polymers, but the limitations of the technique do not allow analysis of small concentrations or high molecular weight biomolecules.[3] Another spectroscopic technique suitable for studying the structural conformations of adsorbed proteins is Raman spectroscopy, and the information provided by this technique is limited to specific residues or specific functional groups of interest.[4] Surface enhanced Raman scattering (SERS) provides information about a residue within 5 Å of the surface on which it is adsorbed due to the Raman enhancement.[5] Circular dichroism (CD) has been applied to the study of conformations of adsorbed proteins but a fundamental limitation of CD, Raman spectroscopy, and SERS is that the contribution of interactions from specific residues often cannot be assigned, thus making spectral interpretation ambiguous. Due to the limitations of these techniques, we decided to use the exquisite specific binding characteristics of antibodies to their respective antigen epitopes to probe the conformations of an adsorbed protein.

The immune system is known to recognize structural characteristics of invading organisms and molecules to elicit a specific immune response in the form of antibodies. These antibodies recognize a specific domain of the molecule known as an epitope. There are two types of epitopes: conformational and sequential. Conformational epitopes (discontinuous epitopes) are formed from residues adjacent in space but not necessarily in the primary sequence, and sequential epitopes (continuous epitopes) are defined by adjacent amino acids in the polypeptide chain.[6] Monoclonal antibodies[7] can provide the necessary homogeneity for a selective epitope probe. If the epitope of these antibodies is in an area of interest, then conformational or orientational dependence of antibody binding can be exploited as the basis for conformational probing. Therefore, the diversity of the immunological repertoire and the specificity of the antibodies to particular epitopes have been combined in this study to investigate the conformation and orientation of adsorbed cytochrome c_3 on a Hg electrode.

The cytochromes c_3 form a unique class of hemoproteins which function in the respiratory chain to facilitate anaerobic sulfate reduction in bacteria of the *Desulfovibrio* genus. The protein mediates the electron transfer between a hydrogenase and iron−sulfur proteins, such as ferredoxin.[8,9] The structural and the physiological properties of four types of cytochrome c_3 have been studied: cytochrome c_3 from *Desulfovibrio vulgaris* strain Hildenborough (DvH), which was used in this study, cytochrome c_3 from *D. vulgaris* strain Miyazaki (DvM), *Desulfovibrio desulfuricans* strain Norway (DdN), and *Desulfovibrio gigas* (Dg). The amino acid sequences and the heme attachment of these cytochromes c_3 are known.[5] The c_3 cytochromes consist of a polypeptide chain to which the hemes are covalently bound through two thioether linkages derived from the eight cysteines. There are two histidine residues for each heme which act as axial ligands, one of which is

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sequentially adjacent to the cysteine that forms the covalent bond with the heme. The other histidine is distant from the heme binding site and acts as the sixth ligand to the heme. This heme−histidine coordination is responsible for the tertiary structure of the cytochrome c_3 molecule.

The net charges of the different cytochromes depend on their p*I* values and the pH of the buffer solution in which it is solubilized. Cytochrome c_3 (DvH) is basic (pI = 10.5)[10] and at pH 7.0, the net charges of the molecule are +8 and +4 for the oxidized and reduced forms, respectively. The charge distribution on the protein surface is asymmetric, and heme 1 is in a positive field because of the high lysine content in its vicinity. The X-ray crystallographic structure of cytochrome c_3 from DvH[11] suggests that the overall dimension of a cytochrome c_3 molecule is approximately 33 × 39 × 34 Å. In the DvH structure, each of the heme groups is located in a different protein environment, and the different protein environment of the hemes may be responsible in part for their different redox potentials. The solvent exposure contact areas of the hemes are 127, 168, 136, and 136 $Å^2$ for hemes 1−4, respectively, and this is a very high degree of exposure when compared with cytochrome c, in which it is typically 32−49 $Å^2$. It is believed that the large heme exposure areas are responsible in part for the low redox potentials, which resemble those of simple model compounds.

The low redox potentials and the high solvent exposure of the hemes have inspired electrochemists to study the electron transfer mechanisms of these molecules. Mercury electrodes have been widely used in the structural studies of heme proteins, due to the good reproducibility of surface properties. Recently, electron transfer studies of cytochrome c_3 from DvH on a Hg electrode[12] have identified three different electrochemical forms (A−C) which are induced by the applied electrode potentials of open circuit, −0.7, −1.2 V, respectively, after the adsorption of cytochrome c_3 on Hg at open circuit potential. The A and C forms are electroactive, and the B form is electroinactive. All of these transitions are irreversible and take place only from A to B, B to C, and A to C. All of the potentials referred to in this paper are referenced to the Ag/AgCl reference electrode. Although the adsorption of cytochrome c_3 on Hg has been described,[13−15] there are no reported definitive studies of the conformation of cytochrome c_3 on the Hg electrode. In the recent studies by Zhang,[16] an attempt was made to establish whether there are spectroscopically detectable structural changes associated with these three electrochemical forms as measured by surface enhanced Raman scattering (SERS). No clearly defined transitions were observed, apparently because the relevant functionalities were not within 5 Å of the surface. In the present study, the possibility of using monoclonal antibodies with epitopes in the vicinity of heme 1 was explored to establish whether the three electrochemically distinct forms corresponded to three structurally distinct conformations of cytochrome c_3 on the Hg electrode. The conformation sensitive nature of the antigen−antibody interaction was used to study conformational changes of adsorbed cytochrome c_3 on Hg and to investigate the orientation of the molecule in the three adsorbed forms. This was accomplished by employing wild type cytochrome c_3 and two mutants (H70M and H70V) in which one of the heme axial ligands is replaced by methionine and valine, respectively. The results of the analysis of orientations and conformations of cytochrome c_3 on the Hg electrode using monoclonal antibodies as probes will serve to demonstrate the utility of monoclonal antibodies to analyze the orientation and conformation of adsorbed protein.

Experimental Section

For materials, apparatus, preparation of monoclonal antibodies, cloning and ascites production, purification of monoclonal antibodies, and immunoreactivity of the antibodies, consult the Supporting Information. Procedures specific to this study are presented below.

Screening of Hybridomas. The hybridomas were screened by a solid phase enzyme-linked immunosorbent assay (ELISA) in which wild type cytochrome c_3 and mutants H70M and H70V were adsorbed on the microtiter plate. The ELISA plates were coated with 100 μL/well of 10 μg/mL solutions of cytochrome c_3 protein in 0.1 M carbonate buffer (pH 9.35) for 2 h at 37 °C. The plates were washed four times with wash buffer (0.01 M phosphate buffer, pH 7.4 containing 0.15 M NaCl (PBS) and 0.05% Tween 20). Then the plates were incubated 1 h with 100 μL/well of ELISA diluent buffer (wash buffer containing 0.2% gelatin) to block the nonspecific adsorption to the plate. After the plates was washed four times, 100 μL of culture supernatants were added and incubated for an additional hour at 37 °C; 100 μL/well of Gt×Mo−IgG−HRP conjugate (1:10000 dilution) was added, after the plate was washed with wash buffer. The plate was incubated for 1 h at 37 °C. Finally, the plates were washed with nanopure water, and 100 μL of tetramethylbenzidine (TMB) solution was added. After 20 min at room temperature, the color reaction was stopped by adding 50 mL of 1 M HCl and the absorption was read at 450−650 nm using a microtiter plate reader.

Porphyrin c_3 Preparation. Porphyrin c_3 was prepared by the method of Flatmark and Robinson.[17] In a Teflon container, 400 μg of cytochrome c_3 was lyophilized. Distilled HF (0.5 mL) was added to the Teflon container, which was in an ice bath, and the protein was dissolved. The purple fluorescent solution was aspirated with N_2 gas for about 5 min to remove the HF. The Teflon container was then placed in a desiccator and dried over NaOH under reduced pressure for 2 h. The protein was dissolved in 1 mL of 6 M guanidine hydrochloride in 0.05 M ammonium acetate buffer, pH 5.0, stirring gently for 4 h at 4 °C. The protein was purified using a Sephadex G-25 column, which was equilibrated with 0.05 M ammonium acetate buffer, pH 5. The porphyrin c_3 fraction was collected, monitoring the eluent at 280 nm. Absorbance spectra were obtained for all the fractions collected. These were comparable to the previously obtained spectra of porphyrin *c*.[17] A small amount of dithionite was added to a solution of porphyrin c_3. There were no changes in the absorption spectrum, confirming that there is no significant amount of iron bound to porphyrin c_3. The porphyrin c_3 fraction was then subjected to HPLC size exclusion chromatography for molecular weight confirmation to verify that the HF treatment did not cleave any bonds in the protein backbone. The molecular weight was confirmed by a calibration curve. The porphyrin c_3 was denatured by heating the sample in a water bath for 5 min at 80 °C.

Reactivity of the Antibodies with Porphyrin c_3 and Heat-Denatured Porphyrin c_3. The concentration of the porphyrin c_3 was calculated using a bicinchoninic acid (BCA) assay and was also verified spectrophotometrically.[17] The apparent binding constants were estimated using the procedure described in the evaluation of the immunoreactivity of the antibodies.

Inhibition ELISA. ELISA plates were coated for 2 h at 37 °C with 100 μL/well of a 10 μg/mL wild type cytochrome c_3 solution in 0.1 M carbonate buffer, pH 9.35. After the plates were washed with wash

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buffer, they were blocked with ELISA diluent buffer for 1 h at 37 °C. In a low protein binding ELISA plate, wild type cytochrome c_3 was serially diluted with ELISA diluent buffer starting at 6.25×10^{-6} M. Then, a constant antibody concentration was added (for 2A2 and 4H8 6.25×10^{-8} and 3.13×10^{-8} M, respectively). After the solutions were mixed using an octapet, 100 μL out of the 200 μL total volume was transferred to the previously blocked plate and incubated 1 h at 37 °C. The monoclonal antibody bound to the plate was determined using a Gt×Mo−HRP conjugate.

Study of Desorption and Conformational Stability of Cytochrome c_3 Electrochemical Forms on Hg Electrode without an Applied Potential. (1) Stability of the Cytochrome c_3 A Form on a Hg Electrode. Cytochrome c_3 was adsorbed on the Hg electrode according to Zhang and Wilson.[12] Briefly, the static Hg drop electrode was immersed in a 3 mM cytochrome c_3 solution for 3 min at open circuit. The electrode was washed with nanopure water and transferred to a 0.03 M phosphate buffer, pH 7.0. Then, the cell was degassed with argon for 4 min, and the electrode was incubated for 4 min at open circuit (∼−0.05 V). The cytochrome c_3 modified Hg drop was then transferred to an ELISA well that contained 0.03 M phosphate buffer, pH 7.0. After six cytochrome c_3 modified Hg drops were obtained, there was a 2 h interval before obtaining the second six drops. Immediately after the twelve drops were obtained, they were incubated with ELISA diluent buffer which contained 0.3% gelatin for 1 h at 37 °C to block nonspecific adsorption. The plates were washed, and 100 mL of 1 mg/mL 2A2 antibody was added and incubated for another hour at 37 °C. The bound antibody was detected by the Gt×Mo−HRP conjugate.

(2) Stability of the Cytochrome c_3 C Form on a Hg Electrode. Cytochrome c_3 was coated on the Hg drops as described above. After the cell was degassed with argon for 4 min, the adsorbed cytochrome c_3 was incubated at a cathodic potential of −1.2 V for 4 min. Then, a cyclic voltammogram was immediately obtained, and the same cytochrome c_3 modified Hg electrode was kept in the cell under a steady stream of argon for 3 h at open circuit. Immediately thereafter, another cyclic voltammogram was obtained.

Characterization of Cytochrome c_3 on the Hg Electrode. Cytochrome c_3 was adsorbed on a Hg electrode using the methods described in the time-dependent study. After the electrodes were incubated at open circuit, Hg drops were washed and transferred to a microtiter well which contained 0.03 M phosphate buffer, pH 7.0. The phosphate buffer was aspirated, and 100 μL of ELISA diluent buffer containing 0.3% gelatin was added and incubated for 1 h at 37 °C. The normal ELISA procedure was followed with serial dilution of MAb, with the following modifications: Gt×Mo−HRP was diluted to 1:15000 and the substrate incubation time before adding the stop solution was increased to 30 min. The same procedure was followed for the characterization of antibody affinities for cytochrome c_3 modified Hg drops which were incubated at −0.7 and −1.2 V.

Mercury Drop Radioimmunoassay. Cytochrome c_3 adsorbed Hg electrodes were incubated for 4 min under argon, in 0.03 M phosphate buffer at open circuit, −0.7 V, −1.2 V, respectively. These cytochrome c_3 coated Hg drops were then transferred to an ELISA well that contained 0.03 M phosphate buffer, pH 7.0. The ELISA was similar to the procedure described for the determination of the apparent binding constant ELISA for antibodies, with the exception that 100 μL of ^{121}I-labeled Gt×Mo was used after diluting the original solution 100 times. Hg drops were transferred to test tubes after washing the drops four times with nanopure water to read the radioactivity using the γ-counter. The control wells were treated similarly except ELISA blocking buffer was added instead of antibody solution.

Results and Discussion

Selection of Hybridomas. A key to the success of the described approach is the screening of the hybridomas immediately after the fusion, to identify the best cell line producing an antibody of well-defined characteristics.[18] The antibodies employed must have certain properties in order to probe protein orientation and conformational change. Since the heme 1 region is of particular interest, all antibodies must bind to non-native conformations of the protein induced by adsorption on the electrode surface. In addition to these two properties, antibodies must be selected which can detect large and small conformational changes, respectively. An antibody which binds to a conformational epitope will be most sensitive to conformational changes. The sequential epitope antibody, although less sensitive to conformational change, can be used to demonstrate that the epitope is still present, i.e., the antigen has not desorbed or reoriented so that the epitope is no longer exposed. To achieve this goal, the structural characteristics of heme 1 and the structural changes of cytochrome c_3 after site-directed mutagenesis were exploited in the screening process. As will be demonstrated, these antibodies react poorly or not at all with the solution form of cytochrome c_3.

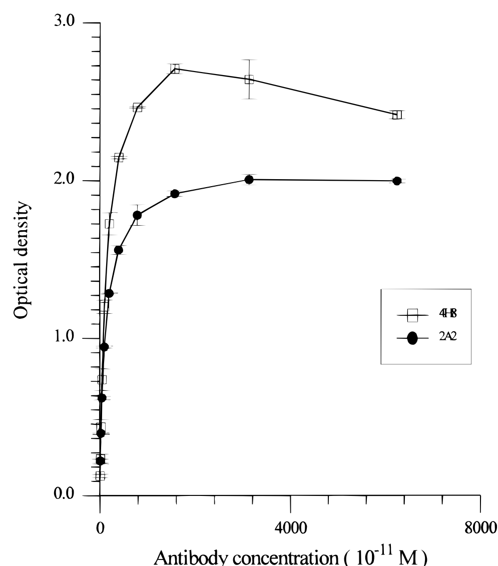

Figure 1. Titration curve for antibodies 2A2 and 4H8 to wild type cytochrome c_3.

Table 1. Apparent Binding Constants of 2A2 and 4H8 to Cytochrome c_3 Wild Type, H70M, and H70V

antibody (subisotype)	apparent binding constant (M^{-1})		
	wild type	H70M	H70V
2A2 (IgG1)	1×10^9	$<10^6$	$<10^6$
4H8 (IgG3)	1×10^9	5.5×10^6	2.6×10^8

Apparent Binding Constant Determination. The apparent binding constants for 2A2 and 4H8 antibodies were calculated for wild type cytochrome c_3, H70M, and H70V by taking the reciprocal of the concentration of the antibody that showed half of the maximum response.[19] The typical response vs antibody concentration for these two antibodies to wild type cytochrome c_3 is shown in Figure 1. A comparison of the apparent binding constants for the wild type, H70M, and H70V is summarized in Table 1. Significant differences in the apparent binding constants between the wild type and the two mutants were strong evidence that the epitopes of these antibodies are in the vicinity of heme 1. Apparent binding constants of the 2A2 antibody to the two mutants were 3 orders of magnitude lower than that of the wild type. Significant change in the binding constant to H70V could be due to the sensitivity of the 2A2 antibody to the structural changes imposed by the inability of the valine to

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coordinate with the Fe in heme 1. Although previous studies using NMR and molecular modeling have confirmed that there are no significant structural changes in the H70M mutant,[20] the reduction of the binding constant of H70M suggests that the 2A2 antibody is also sensitive to the amino acid residue at position 70. The drastic change in 2A2 antibody binding to the two mutants suggests that this antibody is sensitive to an extremely small conformational change in or around histidine 70 and in the vicinity of the heme 1. The two binding constants for the mutants provide strong support for 2A2 recognition of a conformational epitope.

Although the apparent binding constant of the 4H8 antibody to H70V mutant is only an order of magnitude lower than that for the wild type cytochrome c_3, the ability of the 4H8 antibody to bind to the wild type and to the H70V mutant with high apparent binding constants suggests that the epitope recognized by this antibody is not sensitive to the conformational change imposed by H70V. Reduction of the apparent binding constant by 2 orders of magnitude to H70M supports the importance of the histidine 70 to this epitope and shows that the orientation of the methionine residue, when it is coordinated to heme 1, hinders the binding of the 4H8 antibody. These binding constants strongly suggest that the 4H8 antibody recognizes a sequential epitope on the cytochrome c_3 molecule.

Characterization of the Binding Domains of 2A2 and 4H8. From the binding studies, it was suggested that the 2A2 and 4H8 antibodies were specific to a conformational and a sequential epitope, respectively. To further establish the binding domains of these antibodies, porphyrin c_3 was prepared according to the porphyrin c preparation method of Flatmark and Robinson.[17] It has been confirmed that porphyrin cytochrome *c* has a compact structure similar to native cytochrome *c*, although it is less stable to heat denaturation.[21] Assuming that the porphyrin c_3 structural characteristics are similar to porphyrin *c*, porphyrin c_3 facilitated the investigation of the conformational epitope due to the partial denaturation of the protein.

The apparent binding constants were determined using a normal binding ELISA in which the porphyrin c_3 was adsorbed on the microtiter plate. The apparent binding constant for the 4H8 antibody was 2×10^6 M^{-1} and the 2A2 antibody did not show any binding. The apparent binding constant for the 4H8 antibody to heat denatured porphyrin c_3 protein was 3.3×10^6 M^{-1}. The binding patterns of these antibodies could be explained by the structural differences between wild type cytochrome c_3 and porphyrin c_3. Due to the absence of the iron in porphyrin c_3, the histidine residues cannot coordinate with the appropriate hemes. This creates a small structural change in the cytochrome c_3 molecule, and the porphyrin c_3 probably assumes a partially denatured structure around the coordinating histidines.

The moderate binding constant of 4H8 antibody to porphyrin c_3 confirms the ability of this antibody to bind to a non-native protein. The binding ability of this antibody to denatured porphyrin c_3, which was generated by heating the sample to 80 °C, confirmed the epitope of this antibody. Comparing the presumed structural differences between the heat denatured porphyrin c_3 and the wild type and the associated variation of the binding constants between the wild type and the two mutants, it is concluded that the epitope of the 4H8 antibody is sequential. The binding constants to the mutants also show that the histidine 70 residue is an essential component in this sequential epitope.

The inability of the 2A2 antibody to bind to the porphyrin c_3 confirms the sensitivity of the 2A2 antibody binding to the denaturation of the protein. The 3 orders of magnitude reduction in the 2A2 binding constants for the two mutants and the inability to bind to porphyrin c_3 confirm that this epitope is conformational. The binding to the H70M mutant also implies that this conformational epitope encompasses the histidine 70 residue.

The binding pattern of both 2A2 and 4H8 antibodies with wild type cytochrome c_3, porphyrin c_3, and the mutants confirms that the epitopes for these antibodies are in the vicinity of heme 1. Thus, these antibodies are able to recognize the structural changes in cytochrome c_3 on the electrode, if the heme 1 region is accessible.

Binding Characteristics of 2A2 and 4H8 to Solution Phase Cytochrome c_3. The antibodies were further studied to characterize their binding to soluble cytochrome c_3. These experiments helped to understand the accessibility of the epitopes in native cytochrome c_3 to the antibodies and the structural changes that occur when cytochrome c_3 is adsorbed on a surface such as an electrode or microtiter plate. It has been well established that denaturation of protein occurs when adsorbed on a microtiter plate[22] and to the Hg electrode surface.[23−26]

An inhibition ELISA was done to verify the binding of these antibodies to cytochrome c_3 in solution. The results of the inhibition ELISA (see Supporting Information Figure 4) reveal that the 4H8 antibody does not recognize the cytochrome c_3 in solution even at an antibody/cytochrome c_3 ratio of 1/100. The 4H8 antibody has a sequential epitope, and if this epitope were exposed in the native cytochrome c_3, this should inhibit the binding of the antibody to the adsorbed form. This confirms that the epitope of 4H8 is only exposed when the cytochrome c_3 is in a denatured form.

The 2A2 antibody curve shows that there is a small inhibition (8.3%) when the antibody/cytochrome c_3 ratio is 1:50. The ability of the 2A2 antibody to interact weakly with the solution phase cytochrome c_3 shows that the 2A2 antibody could recognize some similarities in the epitopes of the solution and adsorbed forms of the protein. The low inhibition of the native form and the high binding to the adsorbed protein on the microtiter plate confirmed that the epitopes recognized by both these antibodies are fully exposed when cytochrome c_3 is adsorbed. These results also indicate that the cytochrome c_3 is in a denatured form when adsorbed to the microtiter plate.

Is it Possible To Study Cytochrome c_3 Conformations on Hg Electrode Using Antibodies as Probes? The structural changes induced by cytochrome c_3 adsorption on the Hg electrode at different applied potentials can be studied if a particular adsorbed form remains stable and does not desorb from the electrode during the associated immunoassay. This poses two major obstacles when antibodies are used as the structural probes. The first obstacle is the presence of disulfide bonds in the antibody molecule. There are at least 30 disulfide bonds in a monoclonal antibody molecule, and these have redox potentials around −0.43 V.[27] Therefore, the use of antibodies to study the structural differences at an applied potential lower

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than −0.43 V will result in disulfide bond reduction leading to a high nonspecific response due to the adsorption of reduced disulfides on the surface of the Hg electrode. The second obstacle relates to the time required to perform an immunoassay. Normally, an ELISA procedure takes at least 3−4 h. To perform a large number of ELISAs on each modified Hg electrode at the dropping mercury electrode (DME) will take a significant amount of time. The results obtained by performing such an ELISA with one Hg electrode at a time cannot be compared due to the repeated preparation of conjugate and substrate solutions for each modified Hg electrode and to the instability of these solutions.

These obstacles were overcome by performing the ELISA on a microtiter plate by transferring the modified Hg drops to the plate wells and performing the ELISA for all the Hg drops simultaneously. However, prior to this assay, the stability of adsorbed cytochrome c_3 against desorption and the conformational stability for at least 3 h in the absence of an applied potential (the time required to perform an ELISA) was established. Accordingly, cyclic voltammetry was used to characterize the adsorbed film immediately after the modification and then again after 3 h. If both voltammograms show the same redox potentials and peak height, then conformational stability and nondesorption of adsorbed cytochrome c_3 during the interval is likely.

Stability of A Form without an Applied Potential. It is known that the A form of cytochrome c_3 transforms into the B form if repetitive cyclic voltammograms are performed between −0.2 and −0.8 V. The electrochemically inactive B form will reduce the peak current of the cyclic voltammogram in repeated scans. Due to this property, the electrochemical method could not be used to study the stability of the A form. The stability studies were performed according to the methods described in the Experimental Section, the average responses obtained for both columns of the microtiter plate were equal, and the coefficient of variation (CV) between the two columns were less than 2.5%. The CV of 2.5% is acceptable when compared with the standard acceptable value of a normal ELISA which is around 5%.[28] Similar averages for both columns confirm that the adsorbed cytochrome c_3 in the A form does not desorb in 3 h and that the A form is stable in the absence of an applied potential. Conformational stability could be explained by the behavior of the 2A2 antibody. The 2A2 antibody has a conformational epitope, and if the conformation of the A form varied with time due to the lack of an applied potential, then the response of the two columns on the microtiter plate that contained the modified Hg drops with the A form of cytochrome c_3 obtained at different times would have changed drastically. These changes were not observed, and thus, the adsorbed A form was confirmed as stable for 3 h at open circuit.

Stability of C Form without an Applied Potential. It has been established that the C form on the Hg electrode is stable for at least 20 continuous scans between −0.2 and −0.8 V.[16] Using this property, the stability of the C form without an applied potential for 3 h was studied using the electrochemical method. The cyclic voltammogram immediately after adsorption and the peak potentials and currents in a voltammogram 3 h later without an applied potential were compared. Identical peak currents in both voltammograms confirm the minimal desorption of electroactive species from the electrode. Redox potential stability confirms the stability of the C form on the Hg electrode without an applied potential.

The nondesorption and the stability of the A and C forms of cytochrome c_3 on a Hg electrode without an applied potential illustrate the ability to study conformational analysis using an immunoassay. The stability of the B form was not studied but assumed as stable by considering the amount of cytochrome c_3 adsorbed to the Hg electrode, which fell between that of the A and C forms. Both forms were stable for more than 3 h.

Apparent Binding Constants to the A Form of Cytochrome c_3 on a Hg Electrode. The apparent binding constants of these antibodies to the A form of cytochrome c_3 on the mercury electrode were obtained using an ELISA (see Supporting Information Figure 5). The apparent binding constants were 3.3×10^8 and 3.3×10^8 M^{-1} for 2A2 and 4H8, respectively. The binding constants for the A form on Hg are 1 order of magnitude lower than for binding to cytochrome c_3 on the microtiter plate. These results confirm the existence of a small structural difference between the A form of cytochrome c_3 on Hg and the cytochrome c_3 adsorbed on the microtiter plate.

Orientation of Heme 1 on the Hg Electrode in Cytochrome c_3 A Form. It has been previously hypothesized by Bianco et al.[14] that the physical area of the protein adsorbed onto the mercury electrode will depend on the potential of zero charge (pzc) of Hg and the charge of the protein molecule. The potential of zero charge of the Hg electrode in a phosphate buffer is −0.4 V at pH 7.0,[29] and cytochrome c_3 is positively charged at pH 7.0 due to its high p*I* value (p*I* = 10.5). There are large number of lysine residues[11] in the vicinity of heme 1, and at pH 7.0, these lysine residues are positively charged. If Bianco's hypothesis, based on electrostatic arguments, is correct, then the cytochrome c_3 adsorbed on the Hg at open circuit ($E_{app} \approx -0.05$ V) will orient so that the heme 1 is facing the solution rather than the electrode surface. Both antibodies characterized in this study show binding to an epitope in the vicinity of the heme 1. The high apparent binding constants of these antibodies to the A form on the Hg electrode confirm that the cytochrome c_3 orientation on Hg does not block the binding epitope, established to be in the heme 1 region. The ability of these antibodies to bind to heme 1, confirms that heme 1 on Hg at open circuit is facing the solution rather than the surface of the electrode, thus confirming part of the hypothesis of Bianco.

Is the A Form of Cytochrome c_3 on the Hg Electrode Native or Denatured? Electrochemical studies of cytochrome c_3, have established strong adsorption of cytochrome c_3 (DvM) on the Hg electrode.[13,30] Although it is strongly adsorbed, the extent of deformation of cytochrome c_3 is considered very small.[15] These deformation studies were based on the amount of cytochrome c_3 adsorbed on the Hg electrode, the area of the electrode, and the calculated cross sectional area of the cytochrome c_3 molecule from X-ray crystallographic studies. Nevertheless, this method does not provide any direct physical evidence in favor of or against cytochrome c_3 denaturation on the Hg electrode.

The binding study of 4H8 antibody to the A form enables analysis of the interaction of cytochrome c_3 with a Hg electrode. The inhibition ELISA confirmed that the epitope recognized by the 4H8 antibody is a denatured form of cytochrome c_3, and this epitope is not apparent in the native (solution) form. The

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Table 2. Results of Conformational Analysis of Cytochrome c_3 on Mercury Electrode using Radioimmunoassay

	incubation potential		
	open circuit (0.05 V)	−0.7 V	−1.2 V
conformation	A	B	C
surface coverage (mol/cm^2, 10^{-11})	1.10 ± 0.05	0.94 ± 0.2	0.67 ± 0.1
surface coverage ratio[a]	1.64 ± 0.15	1.40 ± 0.25	1
2A2 antibody response ratio[b] (n = 3)	6.70 ± 0.08	1	NA[c]
4H8 antibody response ratio[b] (n = 3)	6.86 ± 0.3	3.90 ± 0.32	1

[a] Surface coverage of X form/surface coverage of C form. [b] Response of X form/response of C form. [c] NA, no response.

3.3×10^8 M^{-1} apparent binding constant of 4H8 to the A form on the electrode confirms that the A form is a denatured form of cytochrome c_3.

Conformational Analysis of Cytochrome c_3 on Hg Electrode Using Radioimmunoassay. To verify the existence of three conformations on the Hg electrode induced by incubation potentials, a radioimmunoassay was performed. The antibody concentrations for both 2A2 and 4H8 used in this assay were selected from the titration curves of the A form. An antibody concentration of 5 μg/mL was used for the 2A2 antibody, because it lies on the top of the rising portion of the binding curve. The 4H8 antibody concentration selected was 40 μg/mL, as this concentration also lies on the plateau of the binding curve.The careful selection of these conditions was very important in this study. The highest concentration on the rising portion of the antibody concentration vs response curve shows that antibody binding to adsorbed protein can reach saturation on the electrode. It has been reported that the three forms that adsorb on Hg electrode contain different amounts of cytochrome c_3, ranging from 100 to 200 fmol/Hg drop or $0.67-1.1 \times 10^{-11}$ mol/cm^2.[12] The concentrations of adsorbed cytochrome c_3 are reduced when passing from the A to the C form. The following assumption was made to confirm the existence of these three conformations. If all of these forms have the same structural characteristics, then the response should change linearly with adsorbed concentrations of cytochrome c_3 in comparison to the A form. If the responses do not change linearly with the cytochrome c_3 concentrations at these three potentials, then there must be structural differences between the three forms of cytochrome c_3 on the Hg electrode.

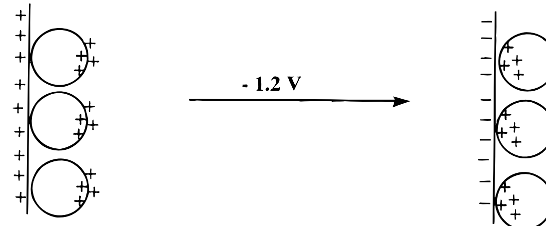
The results of the RIA are shown in Table 2. They show that although the measured surface coverage decreases by a factor of 1.4 in passing from form A to B, the amount of antibody binding decreases by a factor of 6.7. If the structural characteristics for A and B forms were similar, then the response should change with a ratio equal to the concentration ratio. The greater response change for binding compared to the change in the amount of cytochrome c_3 on the electrodes confirms different structural characteristics for the A and B forms. The response ratio of 4H8 antibody for these forms are 6.68:3.9:1 when the surface coverage ratio of these three forms are 1.64:1.4:1. The differences in these ratios also show different antibody binding patterns. The higher response ratio change is due to the lower binding constants for the B and C forms compared to A. This also indicates that the C form has the lowest binding constants. Thus, the existence of at least three structurally different cytochrome c_3 conformations on a Hg electrode can be established.

Although there are clearly three structurally different conformations, the response change could be due to the A form remaining on the electrode without undergoing the structural change to B and C forms, but binding to the antibodies to cause a response. If this were true, then the amount of A form in B and C forms should be equal in both experiments and the response ratios for both antibodies should be equal. The different response ratios for 2A2 and 4H8 antibodies confirm that the response is due to the binding of the antibodies to structurally different conformations.

Table 3. Apparent Binding Constants for Cytochrome c_3 A−C Forms

incubation potential	apparent binding constant (M^{-1})		
	open circuit (∼0.05 V)	−0.7 V	−1.2 V
form	A	B	C
2A2	3.3×10^8	5×10^5	$<10^5$
4H8	3.3×10^8	3.2×10^6	4×10^5

Pathway 1.



Pathway 2.

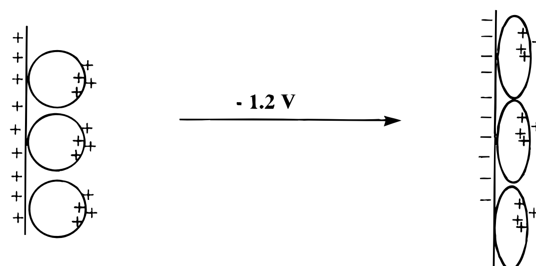


Figure 2. Proposed pathways for the transformation of adsorbed cytochrome c_3 from the A form to the C form.

Apparent Binding Constants of 2A2 and 4H8 to B and C Forms. Although the RIA confirmed the existence of three conformations, this could be independently reconfirmed by studying the binding constants of the 2A2 and 4H8 antibodies to the B and C forms. The binding constants of these antibodies will differ from form to form if there are conformational changes associated with the electrochemical forms observed in the electrochemical study.[16] The results for the binding constant studies are summarized in Table 3. The binding constants for 2A2 and 4H8 antibodies showed a decreasing affinity to the electrochemical forms going from the A to the C form. The existence of different binding constants for both antibodies to the A and to the C form confirmed the three structurally independent cytochrome c_3 forms.

Does Cytochrome c_3 Adsorbed on a Mercury Electrode Reorient at an Incubation Potential of −1.2 V? The binding constants for the cytochrome c_3 A form confirmed that at open circuit the protein orients in such a way that the positively charged heme 1 area is facing the solution. Therefore, the incubation potential of −1.2 V, which is more negative than the pzc of Hg, should attract the positively charged heme 1 toward the electrode surface. The transformation of the heme 1 toward the electrode could be assumed to occur by two pathways as shown in Figure 2. In the first pathway, the adsorbed cytochrome c_3 could desorb from the electrode and

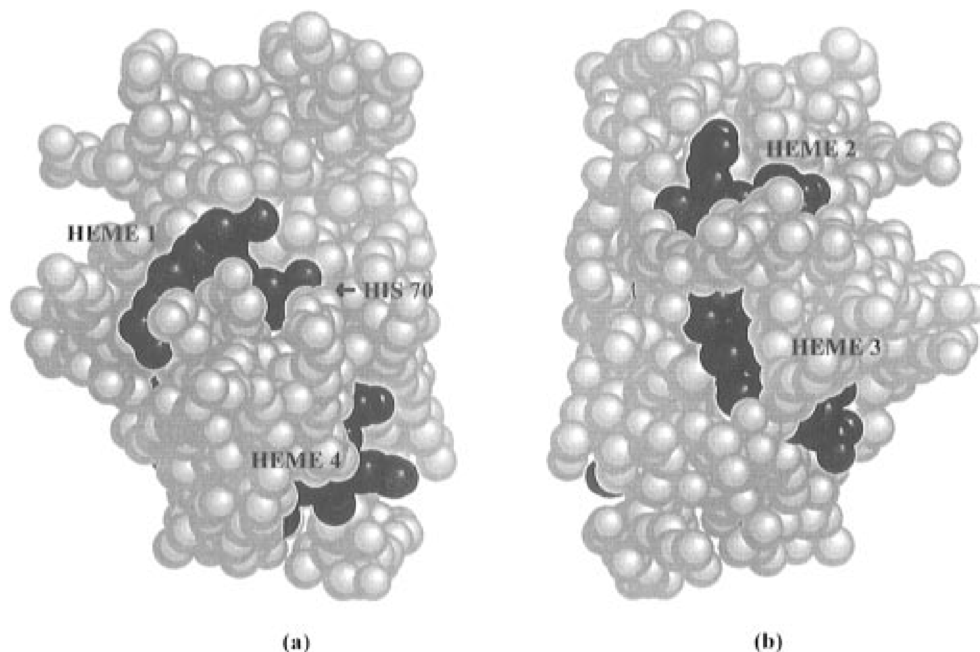


Figure 3. Space-filling model of cytochrome c_3 (Hildenborough) adapted from the X-ray structure.[11] (a) View of side of molecule facing solution. (b) View of side of molecule facing electrode.

reorient with heme 1 directly adsorbed onto the surface of the electrode. The reduction of cytochrome c_3 on the electrode surface and the change in electrochemical characteristics from an electroinactive to an electroactive state supports this pathway. In the second pathway, cytochrome c_3 does not change its orientation (heme 1 remains oriented toward the solution) but instead flattens out. This process could be facilitated by the electrostatic attraction of the negatively charged electrode and the positive charge of the heme 1 region. If the first proposed pathway is correct, then cytochrome c_3 at an incubation potential of −1.2 V orients such that the antibody cannot react with heme 1. The binding constant studies show that both 2A2 and 4H8 antibodies bind to the cytochrome c_3 C form. The apparent binding constants and the epitopes of these antibodies, which are in the vicinity of the heme 1, support the second pathway. This confirms that, although the electrode is highly negatively charged at −1.2 V, the adsorbed cytochrome c_3 does not undergo a reorientation at the electrode surface.

Molecular modeling of the docking of cytochrome c_3 with a planar surface indicates that hemes 2 and 3 are close to the electrode surface when heme 1 is facing the solution. Figure 3a shows the orientation of hemes 1 and 4 toward the solution such that binding to the specific antibodies is facilitated. Figure 3b indicates that hemes 2 and 3, which are on the opposite side of the molecule, might contact the electrode. On the basis of the electrostatic interactions which might be possible as the negative charge on the electrode is increased, it might be expected that heme 2 would be in more intimate contact at more positive potentials, while heme 3 would contact at more negative potentials. It is tempting to suggest that there is an intermediate state, corresponding to Form B, where there is little or no heme contact with the electrode surface.

Conclusions

The two monoclonal antibodies prepared against DvH cytochrome c_3 were used successfully to confirm the existence of three conformations of cytochrome c_3 on the Hg electrode at three different incubation potentials, as also reflected in the different electrochemical properties observed for this protein at these three incubation potentials.[12]

This is the first reported study of protein adsorption on an electrode using antibodies as probes. The ability to study conformational changes on a mercury electrode containing 100−200 fmol of protein on an area of 0.018 cm^2 using RIA and ELISA provides a new tool for the study of conformational changes of proteins adsorbed on charged interfaces. This study explicitly shows that detection of an adsorbed protein with three different structural characteristics, on a surface of very small area at a low concentration, is possible without specially designed or expensive instrumentation.

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Supporting Information Available: Experimental details, inhibition assay graph, and titration curve (5 pages). See any current masthead page for ordering and Internet access instructions.

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