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Determination of the Orientation of Adsorbed Cytochrome *c* on Carboxyalkanethiol Self-Assembled Monolayers by In Situ Differential Modification

Jishou Xu[#] and Edmond F. Bowden*

Contribution from the Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695-8204

Received June 26, 2005; E-mail: edmond_bowden@ncsu.edu

Abstract: The contact domain utilized by horse cytochrome *c* when adsorptively bound to a $C_{10}COOH$ self-assembled monolayer (SAM) was delineated using a chemical method based on differential modification of surface amino acids. Horse cytochrome *c* was adsorbed at low ionic strength (pH 7.0, 4.4 mM potassium phosphate) onto 10 μm diameter gold particles coated with $HS(CH_2)_{10}COOH$ SAMs. After in situ modification of lysyl groups by reductive Schiff-base methylation, the protein was desorbed, digested using trypsin, and the peptide mapped using LC/MS. Relative lysyl reactivities were ascertained by comparing the resulting peptide frequencies to control samples of solution cytochrome *c* modified to the same average extent. The least reactive lysines in adsorbed cytochrome *c* were found to be 13, 72, 73, 79, and 86−88, consistent with a contact region located up and to the left (Met-80 side) of the solvent-exposed heme edge (conventional front face view). The most reactive lysines were 39, 53, 55, and 60, located on the lower backside. The proposed orientation features a heme tilt angle of approximately 35−40° with respect to the substrate surface normal. Factors that can complicate or distort data interpretation are discussed, and the generality of differential modification relative to existing in situ methods for protein orientation determination is also addressed.

Introduction

Understanding protein orientation at solid/aqueous interfaces is critical to a number of scientific and technological topics, including protein electrochemistry,[1] enzyme immobilization,[2] biomaterials,[3] biosensors,[4] and bioseparations.[5] In the field of protein electrochemistry, cytochrome *c* stands as the most well-studied example of an adsorbed protein with many examples of electroactive monolayers and submonolayers having been reported for COOH-terminated self-assembled monolayers (SAMs)[6−12] and conductive metal oxides.[13−15] Nonetheless, despite this prominent role in protein electrochemistry and the availability of nonelectrochemical cytochrome *c* heme orientation information,[16−21] a well-defined binding site on cytochrome *c* has not yet been delineated at any electrode surface. For the most part, this state of affairs can be attributed to well-known difficulties associated with making in situ orientation measurements on surface-confined proteins, notably the limited quantities of protein that are possible and the presence of property distributions that arise from the heterogeneous nature of surfaces and binding interactions. In the present work, our goal was to delineate the binding site of horse cytochrome *c* (HCC) when adsorbed on $HS(CH_2)_{10}COOH$ SAM-modified gold electrodes.

[#] Present address: Rosemount Analytical, 2400 Barranca Parkway, Irvine, CA 92604.

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Among small proteins, cytochrome *c* has received considerable attention in surface orientation studies due to its appealing spectroscopy (monoheme) and electrochemistry (outer sphere one-electron transfer), its advanced state of structural characterization, and its well-known binding properties. In an influential series of papers, Saavedra and co-workers[16] described quantitative spectroscopic determinations of cytochrome *c* heme orientation at nonconductive solid substrates using two techniques: linear dichroic absorption in attenuated total reflection waveguide geometry and total internal reflection fluorescence (TIRF). The use of two independent measurement techniques enabled determination of both heme orientation (θ, the mean tilt angle) and its angular distribution (θ dispersion) for cytochrome *c* adsorbed to a variety of surfaces, including thiol-, hydroxy-, sulfonate-, and methyl-terminated self-assembled monolayers (SAMs), arachidic acid Langmuir−Blodgett (LB) films, hydrophilic glass, and silicon oxynitride. Adsorption states that ranged from highly oriented ($\theta = 46 \pm 6°$ for arachidic acid LB films) to largely disordered ($\theta = 12 \pm 33°$ for hydrophilic glass) were found. TIRF measurements of heme orientation have also been reported for adsorbed cytochrome *c* at tin oxide, indium−tin oxide (ITO), and glass substrates by Fraaije et al.[17] and Bos and Kleijn[18] and for thiol-, thiol/methyl-, and thiol/hydroxy-terminated SAMs by Blasie and co-workers.[19] Other notable spectroscopic measurements of cytochrome *c* heme orientation were reported by Van Duyne and co-workers for carboxyl-terminated SAMs on silver using surface-enhanced resonance Raman spectroscopy (SERRS)[20] and by Chang and co-workers for bare and modified fused silica using Soret band total internal reflection absorption (TIRA).[21]

Despite the unprecedented insights to cytochrome *c* orientation that continue to be provided by heme-based spectroscopy, the identification of a binding site of high definition is one outcome that is generally unattainable given the usual symmetry assumptions that are made.[16b,17] Rather, a continuum of possible binding states can be identified that are related to each other by rotation about the heme plane normal. Thus, even with the well-established notion of cytochrome *c* binding to anionic partners through its lysine-rich solvent-exposed heme edge face, it seems that considerable latitude would prevail in attempting to identify a specific binding state on the basis of heme tilt angle. Moreover, in the case of TIRF measurements of heme orientation, one must also consider that the protein being characterized is not cytochrome *c* itself, which is nonfluorescent, but rather porphyrin cytochrome *c* or Zn cytochrome *c*. A final limitation of spectroscopic techniques is the requirement of optically compatible substrate materials. For example, the total internal reflection and linear dichroic methods cited above require bulk substrate solids that are optically transparent, thus precluding the use of most electrode materials with the exception of some conductive metal oxides.[17,18] A notable exception is indium−tin oxide (ITO), which has recently been incorporated into planar optical waveguides.[22] Metallic electrodes, on the other hand, are ordinarily limited to techniques based on externally reflected light, such as SERRS.

In the present work, we undertook a chemical strategy for binding site determination based on in situ differential chemical modification of surface amino acids (typically lysines or aspartates/glutamates). This principle was first described by Bosshard and co-workers in seminal studies of the contact domains between cytochrome *c* and various protein partners.[23−26] For a surface-bound protein, exposed residues are expected to be readily accessible to diffusing reagents, whereas spatial exclusion and diffusional resistance should restrict reagent access to those residues involved in interfacial binding. Thus, a protein that prefers to bind in a highly oriented state should give rise to a definitive pattern of differential reactivity relative to the solution state. On the other hand, only a weak or nonexistent differential pattern would be expected for a protein that immobilizes with a weak or completely random orientation. On the basis of its structure,[27−29] we anticipate a strong preferential orientation for horse cytochrome *c* adsorbed on $Au-S(CH_2)_{10}$-COOH self-assembled monolayers. Of its 104 amino acids, 21 are basic (19 lysines, 2 arginines) and 12 are acidic (9 glutamates, 3 aspartates), thus giving rise to nine uncompensated monocationic sites. The 19 lysines are distributed, more or less, over the entire protein surface, whereas the 12 carboxylates are located primarily on the "back face", that is, the surface region facing away from the exposed heme edge. The back face can be considered as approximately neutral, with similar numbers of acidic and basic residues. The majority of the nine uncompensated cationic sites are lysines positioned on the "front face" about the exposed heme edge. Prior studies have clearly established that HCC binds to acidic protein partners through its lysine-rich front face.[30,31]

The methodology that was used relies on the in situ methylation of lysyl groups by reductive Schiff-base reaction with formaldehyde (see Supporting Information). After lysyl modification, HCC is desorbed, enzymatically digested, and analyzed by HPLC interfaced to an electrospray ionization (ESI) mass spectrometer (MS).[32−34] The specific pattern of lysyl modification revealed by peptide sequencing was then analyzed

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to yield a proposed contact domain and adsorption orientation. One complication of this approach is the variability that commonly characterizes lysine reactivity within a protein.[35,36] Accordingly, it is not the absolute extent of methylation of a particular HCC lysyl group that reveals the degree of protection experienced in the adsorbed protein state. Instead, this determination must be made by comparing modified surface samples ($HHC^*_{surface}$) to solution controls ($HHC^*_{solution}$) that display the same average extent of modification. Throughout the text, the use of an asterisk, such as in $HHC^*_{surface}$, indicates a protein, peptide, or lysyl group that has been methylated.

Experimental Section

Chemicals and Materials. Gold particles (~10 μm) of purity >99.95% were purchased from Alfa Aesar. The procedures for cleaning, self-assembly of thiolate SAMs, and adsorption of HCC were essentially the same as those previously described for gold films coated on glass slides except for the absence of electrochemical treatment of gold.[6] 11-Mercaptoundecanoic acid (Aldrich), sequence grade trypsin (Promega), formaldehyde (Aldrich), and $NaBH_3CN$ (Aldrich) were used as received. HCC (Sigma) was purified as previously reported.[37] Two pH 7.2 potassium phosphate buffers (KPB) were prepared at concentrations of 4.4 mM (ionic strength = 10 mM) and 145 mM (ionic strength = 300 mM) and are denoted as KPB(10) and KPB(300), respectively.

Modification Reaction. Thiolated Au particles with adsorbed HCC were thoroughly rinsed with KPB(10) prior to the methylation reaction. In a total volume of 100 mL, 1 g of thiolated Au particles with adsorbed HCC was dispersed in a 50 mL volume of KPB(10) and then mixed with the appropriate amount of freshly prepared 1 M formaldehyde. A 50 mL volume of freshly prepared 10 mM $NaBH_3CN$ solution was immediately added to the mixture. After 5 min at room temperature, the reaction was stopped by pouring the contents into another beaker containing 5 mM lysine (pH 6.5) in 3-fold excess relative to the initial amount of formaldehyde. The mixture was immediately ultrafiltered using an Amicon cell with a YM30 membrane (30 kDa cutoff) followed by three cycles of wash/filtration using 100 mL volumes of 5 mM lysine in 2 mM KPB (pH 7.2). This procedure eliminated unreacted formaldehyde and $NaBH_3CN$ as well as any HCC molecules that may have desorbed during the reaction and the filtration. Modified HCC was then desorbed using a 1:1 mixture of KPB(300) and 0.1 M lysine and separated from the gold particles by ultrafiltration in an Amicon cell with a YM30 membrane. The collected solution was further purified by ultrafiltration with a YM3 membrane (3 kDa cutoff).

LC/MS. Unmodified and modified HCC samples were incubated with sequence grade trypsin (40:1 protein:trypsin ratio, w/w) in a digestion buffer (50 mM, pH 7.8, ammonium bicarbonate) at 37 °C. Unmodified HCC was digested for 6 h. Modified HCC was digested for only 3 h to mitigate the effects of nonspecific tryptic digestion, which is significantly more extensive for modified HCC samples. After halting digestion by adding formic acid, digests were frozen for storage. Thawed samples were dissolved in water at 5−10 pmol/μL. Peptides were separated by HPLC at room temperature on an Xterra MS C18 column (Waters, 5 μm, 2.1 × 100 mm). The solvents were delivered by an HP 1100 binary pump system. The peptides were eluted with 0.2% formic acid/water (solvent A) and 0.2% formic acid + 80% acetonitrile (solvent B) at a flow rate of 0.3 mL/min. The column was preconditioned with solvent A for 15 min. A two-segment linear gradient scheme was employed, increasing B from 0 to 5% for 5 min and then from 5 to 35% for 60 min. HPLC chromatograms generated with UV detection at 280 nm and with MS detection were acquired simultaneously. ESI mass spectra were acquired on a single quadrupole mass detector (HP 1100). Full-scan spectra were acquired in the positive ion mode using ChemStation (HP). The ESI voltage was 3.5 kV with drying gas at 34 psi and 300 °C. The collision voltage of 30 V and the skimmer voltage of 40 V resulted in negligible fragmentation of peptides.

Table 1. Peptides Identified by LC/MS in Tryptic Digests of Native HCC

peptide[a]	sequence[b]	intensity[c]	*m/z*
T1	CH_3CO−GDVEK(5)	5.9	589.5
T2K	GK(7)K(8)	1.1	332.3
T2	GK(7)	0.44	204.2
KT3	K(8)IFVQK(13)	2.2	254.9
3	IFVQK(13)	30	317.9
T4	CAQC(heme)HTVEK(22)	52	409.6
T6	HK(27)	1.7	284.2
T7	TGPNLHGLFGR(38)	71	390.5
KT8	KTGQAPGFTYTDANK(53)	11	533.8
T8	TGQAPGFTYTDANK(53)	11	491.4
T10	GITWK(60)	16	302.6
T11K	EETLMEYLENPKK(73)	7.1	542.4
KT12	K(73)YIPGTK(79)	2.3	269.6
T12	YIPGTK(79)	21	339.7
T13	MIFAGIK(86)	40	390.5
T14T15	TEREDLIAYLK(99)	7.1	451.3
T15	EDLIAYLK(99)	32	483.1
KT16	K(100)ATNE	0.72	572.6
T16	ATNE(104)	0.63	434.2

[a] Peptides in boldface are UV active at 280 nm. [b] Numbers in parentheses are amino acid sequence numbers beginning with N-terminus. [c] Relative mass spectral intensities (arbitrary units) normalized using total UV absorbance at 280 nm (see Supporting Information).

Results

The differential pattern of lysine reactivity for adsorbed horse cytochrome *c* was elicited by comparing samples of adsorbed HCC that were modified in situ while surface-resident ($HCC^*_{surface}$) to control samples of HCC modified to the same average extent while solution-resident ($HCC^*_{solution}$). The three sections below describe the LC/MS characterization of enzymatic digests of native HCC, modified solution HCC controls, and modified adsorbed HCC, respectively. In the Supporting Information, the reader will also find the following results: (1) experiments that document the compatibility of $Au-S(CH_2)_{10}$-COOH SAMs with the formaldehyde/$NaBH_3CN$ reaction conditions; (2) an evaluation of the Schiff-base methylation reaction for a small model compound, N^2-(carbobenzyloxy)-L-lysine; (3) mass spectrometry of native and modified holocytochrome *c*; and (4) LC/MS of native HCC and tryptically digested HCC.

LC/MS of Tryptic Digests of Native HCC ($HCC_{solution}$). Native solution HCC was subjected to tryptic digestion followed by peptide sequencing using LC/MS. Table 1 lists the peptides that were assigned using the protocol described in the Supporting Information. These native peptides cover most of the amino acid sequence of HCC. There are several peptides listed in Table 1 that contain more than one lysine or arginine, an observation indicative of incomplete cleavage at some sites. Two small unresolved peptides, T5 (GGK(25)) and T9 (NK(55)), that have identical masses (260.1 Da) and that eluted with nearly zero retention do not appear in the table. The data in Table 1 also reveal a substantial variation in MS peak intensity among

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peptides, which is largely attributed to differences in ionization efficiency.[38]

LC/MS of Tryptic Digests of Modified Solution-Resident HCC (HCC*$_{solution}$). Samples comprised of HCC that had been modified while in solution (i.e., HCC*$_{solution}$) were subjected to tryptic digestion followed by LC/MS characterization. Analyzing tryptic digests of modified HCC is complicated by the fact that trypsin has much lower activity toward lysyl residues once they have been methylated.[39] For illustration, consider a modified HCC sample that exhibits a low average extent of modification (e.g., ≤5 additional methyl substituents per molecule). An isolated molecule chosen from this sample would typically feature a few lysyl groups that were methylated but with the majority unchanged. At each of the 19 lysine sequence positions, the presence of methyl substitution would therefore be expected in only a minor fraction of the sample. As a result, substantial amounts of unmodified peptides are always observed in digests of lightly methylated HCC samples, albeit at diminished levels relative to native HCC. Also present were new peptides resulting from the coupling of two or more simple peptides at sites of methylation (denoted as "methylated peptides"). After modifying HCC, the total number of unique detectable peptides increased by a factor of 2−3, giving rise to LC/MS chromatograms of greater complexity relative to those for unmodified HCC.

For peptide mapping of tryptic digests of modified HHC samples, one can choose to analyze either unmodified peptides or modified peptides. The average extent of methylation required to optimize the analysis depends on this choice. Thus, to quantify unmodified peptides in modified HCC samples, an average modification extent of 8 methyl groups per molecule ($m = 8$) was used, which significantly lowered unmodified peptide intensities relative to those in native HCC samples. The susceptibility of individual lysyl groups to methylation was also observed to be highly variable. To quantify modified peptides, on the other hand, HCC samples were modified with only 4 methyl groups per HCC molecule ($m = 4$), for which the intensities of methylated peptides are expected to increase monotonically with extent of methylation. For further details on the impact of methylation extent on the analysis of unmodified and modified peptides in solution HHC samples, see the Supporting Information.

Table 2 lists the intensities of *methylated peptides* found in digests of modified HCC solution samples (i.e., HCC*$_{solution}$). Here, the digested sample appears more complex relative to unmodified HHC (see Table 1) because of the fact that a methylated lysyl group can carry either one or two additional methyl substituents. Data analysis is further complicated by the incomplete cleavage that can occur at unmodified lysine residues. Thus, for an arbitrary peptide that happens to contain N lysyl groups in addition to the cleaved lysine or arginine residue, multiple variants of the peptide featuring anywhere from 0 to $2N$ added methyl groups can be present in the sample. The extent of modification of lysyl groups is therefore best represented by the summed intensities of all variants of the peptide. Thus, the total intensity of a methylated peptide, X, is defined as $\Sigma(N \times I_{X,N})$, where $I_{X,N}$ is the intensity of the MS peak due to peptide X with N (= 1, 2, 3, ...) additional methyl groups.

Table 2. Presence of Methylated Peptides in Tryptic Digests of Solution-Modified HCC at Different Extents of Modification (m)[a,b,c]

peptide	$m = 1$	$m = 4$	$m = 8$
T1*T2		0.41	1.1
T2*K		0.63	1.2
K*T3	0.73	1.1	1.4
K*T*3T4		9.5	31
T3*T4	5.1	20	32
T3*T4*T5		1.3	5.9
T4*T5		0.69	1.8
T5*T6		0.26	0.64
T6*T7		1.4	2.5
K*T8		1.0	1.8
T8*T9		0.22	0.45
T9*T10	0.23	0.51	0.91
T10*T11		0.18	0.45
T10*T*11K		1.1	3.3
T11*K		3.3	5.0
K*T12	0.80	0.74	1.6
T13*K		4.3	5.0
K(87)*K		0.93	1.3
K*K*T14		0.27	0.52
T15*K	0.13	1.3	2.2
K*T16	0.13	0.44	0.77

[a] HCC*$_{solution}$ samples modified with 1, 4, and 8 methyl groups per molecule were obtained by reaction with 0.5, 1.75, and 4 mM formaldehyde, respectively. Buffer: 4.4 mM potassium phosphate, pH 7.2. See Experimental Section for additional details of reaction parameters. [b] Tabular values shown are relative mass spectral intensities for HCC modified in solution; see Table 1, footnote c. [c] Extent of modification: m = average number of added methyl groups per HCC molecule.

The results in Table 2 demonstrate that all 19 lysines in HCC*$_{solution}$ samples are susceptible to methylation. The intensities of methylated peptides also show considerable variability. Again, this effect is due to both the variable amounts of different peptides and the variability of ESI-MS detection sensitivity for specific peptides. For the extents of modification used to generate the data in Table 2, most of the detectable methylated peptides were found to contain only one methylated lysyl group. Only four peptides containing two methylated lysyl groups were detected.

LC/MS of Tryptic Digests of Modified Surface-Resident HCC (HCC*$_{surface}$). HCC was adsorbed on Au−S$(CH_2)_{10}$-COOH SAMs and modified in situ as described previously. The average extent of methylation (m) for adsorbed HCC samples was controlled by changing the concentration of formaldehyde while holding the reaction time constant. For each modified sample of adsorbed horse cytochrome *c* (HCC*$_{surface}$), a modified solution control was also prepared at the same value of m by adjusting the formaldehyde concentration. To attain similar values of m, the formaldehyde concentration required by HCC*$_{surface}$ samples was found to be some 2−3 times higher than that required to produce the HCC*$_{solution}$ controls. Thus, there are two factors, namely, formaldehyde concentration (2−3 times greater for adsorbed HCC) and differential chemical protection (adsorbed vs solution states), that will conspire to make the extent of methylation at a particular lysine position in a HCC*$_{surface}$ sample differ from its solution control. Lysyl groups located in the binding domain should be modified less extensively in the surface sample than in the solution control due to better protection. In contrast, exposed lysyl groups positioned away from the binding domain should be modified more extensively in the surface sample due to the higher formaldehyde concentration. Figure 1 shows chromatograms for tryptic digests of a typical HCC*$_{surface}$ sample and its solution

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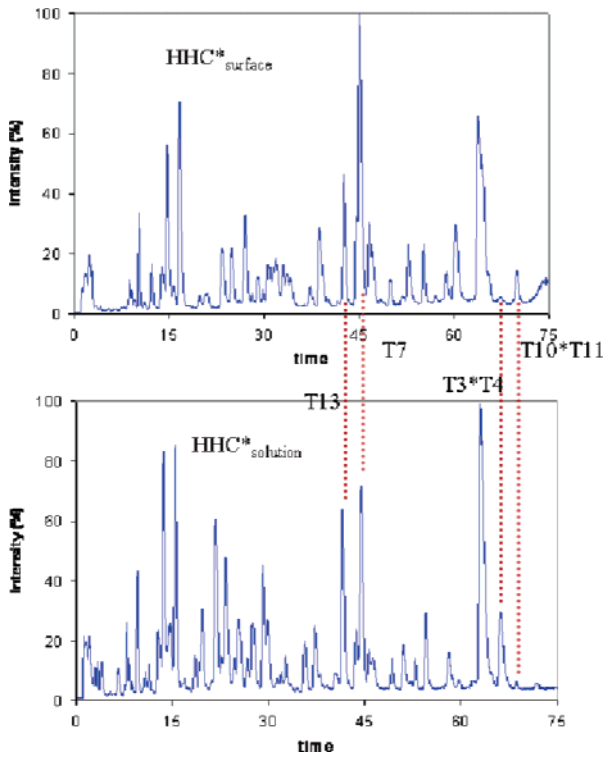

Figure 1. LC/MS chromatograms of tryptic digests of a typical sample of HCC modified while in the adsorbed state (HCC*$_{surface}$) and its solution control (HCC*$_{solution}$). Reaction buffer: 4.4 mM potassium phosphate, pH 7.2. Sample and control were each modified to $m = 4$, that is, an average of 4 additional methyl groups per HCC molecule. Digestion lasted 6 h in a 50 mM, pH 7.8, ammonium bicarbonate buffer at 37 °C containing 40:1 protein:trypsin ratio on a w/w basis.

control. Significant differences are evident. For example, the intensities for the unmodified peptides T7 and T13 are of similar magnitude in the solution control, but the T7 intensity is about twice that of T13 in the HCC*$_{surface}$ sample. Another example is the pair of peaks at 68 and 71 min identified as the methylated peptides T3*T4 and T10*T11, respectively. For the HCC*$_{surface}$ sample, the intensity of the $t = 68$ min peak is negligible and the $t = 71$ min peak intensity is moderate, whereas for the solution control, this relationship is inverted. These and many other differences evident in Figure 1 are proof that disparate patterns of lysine modification result when adsorbed and solution HCC populations are subjected to the same average extent of modification. Mass spectral intensities of modified (methylated) and unmodified peptides identified in adsorbed samples and their solution controls are listed in Tables 3 and 4, respectively.

Discussion

When a protein binds to a surface, the static and dynamic nature of its restricted mobility will be reflected in a characteristic pattern of chemical protection conferred upon surface amino acid residues. To establish the reactivity pattern and elucidate the orientation of cytochrome *c* on Au−S(CH$_2$)$_{10}$-COOH, our main task is to compare lysyl-methylated peptide intensities produced from HCC samples modified in the adsorbed state to those produced from control samples modified in the solution state. The key assumption is that a higher extent of modification for a specific lysine residue results in correspondingly higher signal intensity for any methylated peptide that carries that lysine and correspondingly lower intensity for the unmodified peptide that carries it. Although this assumption works well for HCC samples at limited extent of modification, it is not universally valid.

Binding Face. Figure 2 shows the conventional front and back face views of HCC to be used in this discussion. Table 3 compares the intensities of *methylated peptides* found in digests of HCC*$_{surface}$ samples ($I_{surface}$; column 4) to those found in solution controls ($I_{solution}$; column 3) at $m = 4$ methylation level. The data in columns 3 and 4 are average values obtained from five replicate pairs of surface sample and solution control obtained for a fairly low extent of methylation, namely, an average of 4 methyl substituents per molecule. Column 5 provides the values of the intensity ratio, $I_{surface}/I_{solution}$, which were used to assess relative levels of lysyl protection in the adsorbed state. Smaller values indicate greater protection. An intensity ratio of 1 carries no special significance; it merely indicates a middle-of-the-road level of protection. The differential pattern of protection is graphically depicted in Figure 3, which plots the logarithm of the intensity ratio against the lysine position number. The peptide T10*T11, which carries methylated K60, gave rise to the highest intensity ratio, 13.3. Hence, K60 can be tentatively identified as the lysyl group of adsorbed HCC that is most accessible to incoming reactant, that is, roughly opposite the binding domain. K60 is centrally located on the back face of HCC. At the other extreme, K*T3*T4, which carries methylated K8 and methylated K13, gave rise to the lowest intensity ratio, 0.1. Because T3*T4, which carries only the methylated K13, gave rise to the second lowest intensity ratio, 0.2, K13 appears to be the best protected lysyl group. K13 is located on the front face near the top of the heme group. Thus, the data of Table 3 are at least in qualitative agreement with the accepted view that HCC binds through its lysine-rich front face. These data will be reexamined more closely in the next section.

The average extent of modification (m) is a critical parameter. In our work, a value of 4 methyl substituents per molecule represented a tradeoff between minimizing nonlinearity of the response (see Supporting Information) and maximizing the number of methylated peptides that could be detected. On the right-hand side of Table 4 is a small set of data that was acquired at a lower level of modification, namely, $m = 1.5$. For this sample, the signals for most peptides containing only one methylated lysyl group became too low to quantify. Of the 5 methylated peptides that could be detected in both the HCC*$_{surface}$ sample and its solution control, T3*T4 exhibited the lowest intensity ratio and T10*T11 the highest, an identical result to that obtained at the higher level of modification, $m = 4$. However, if we compare the intensity ratios of these five peptides side-by-side, their relative order is not the same. At $m = 4$, the intensity ratios have the following order: T3*T4 < K*T3 < K*T8 < T4*T5 < T10*T11. At $m = 1.5$, the order is the same except that the third and fourth entries, K*T8 and T4*T5, are interchanged. By assuming that a more accurate reflection of the true state of binding results from lower levels of modification, then we can inquire how excessive modification could possibly distort the intensity ratios determined for T4*T5 and K*T8 to give anomalously large and small values, respectively. For T4*T5, we hypothesize that the level of protection for K22 can be underestimated because of the extremely high susceptibility of K13 to modification when HCC

Table 3. Modified (Methylated) Peptides from Tryptic Digests of Modified Adsorbed HCC Samples (HCC*$_{solution}$) and Modified Solution Controls (HCC*$_{surface}$)[a]

		4 Added Methyl Groups[b,c]			1.5 Added Methyl Groups[b,d]		
modified lysyl	peptide	$I_{solution}$	$I_{surface}$	$I_{surface}/I_{solution}$	$I_{solution}$	$I_{surface}$	$I_{surface}/I_{solution}$
5	T1*T2	2.2 ± 0.2	1.0 ± 0.1	0.5			
7	T2*K	5 ± 1	3.8 ± 0.5	0.8			
8	K*T3	6 ± 1	4.5 ± 0.7	0.8	0.73	0.22	0.30
8, 13	K*T3*T4	134 ± 1	14 ± 1	0.1			
13	T3*T4	194 ± 6	32 ± 5	0.2	5.1	0.27	0.05
13, 22	T3*T4*T5	28 ± 1	10 ± 1	0.4			
22	T4*T5	3.8 ± 0.3	37 ± 2	9.7	1.5	3.9	2.6
25	T5*T6	3.7 ± 0.1	3.5 ± 0.8	0.9			
27	T6*T7	3.6 ± 0.1	20 ± 3	5.6			
39	K*T8	11 ± 1	26 ± 1	2.4	0.10	1.1	11
39, 53	K*T8*T9	3.1 ± 0.1	15 ± 1	4.8			
53	T8*T9	1.5 ± 0.1	3.9 ± 0.8	2.6			
55	T9*T10	0.8 ± 0.1	2.8 ± 0.1	3.5			
60	T10*T11	2.1 ± 0.4	28 ± 3	13.3	0.10	1.8	18
60, 72	T10*T11*K	2.0 ± 0.3	8.2 ± 1	4.1			
72	T11*K	26 ± 1	10 ± 0.8	0.4			
73	K*T12	5 ± 1	2.8 ± 0.1	0.6			
79	T12*T13	5 ± 2	2.6 ± 0.8	0.5			
86	T13*K	23 ± 1	9 ± 1	0.4			
87[#]	K*K	1.3 ± 0.1	0.57 ± 0.4	0.4			
88	K*T14	1.0 ± 0.1	0.4 ± 0.1	0.4			
99	T15*K	15 ± 1	10 ± 1	0.7			
100	K*T16	4.9 ± 0.1	7.7 ± 0.5	1.6			

[a] HCC*$_{surface}$ samples modified with 1.5 and 4 methyl groups per molecule were obtained by reaction with 2 and 6 mM formaldehyde, respectively. HCC*$_{solution}$ samples modified with 1.5 and 4 methyl groups per molecule were obtained by reaction with 0.75 and 1.75 mM formaldehyde, respectively. Buffer: 4.4 mM potassium phosphate, pH 7.2. See Experimental Section for additional details of reaction parameters. [b] Extent of modification: m = average number of added methyl groups per HCC molecule. [c] Values shown for $I_{solution}$ and $I_{surface}$ are average intensities and standard deviations for five replicate experiments. [d] Values shown for $I_{solution}$ and $I_{surface}$ are intensities for a single experiment.

Table 4. Unmodified Peptides from Tryptic Digests of Modified Adsorbed HCC Samples (HCC*$_{solution}$) and Modified Solution Controls (HCC*$_{surface}$)[a]

peptide	lysine	$I_{solution}$[b]	$I_{surface}$[b]	$I_{surface}/I_{solution}$
T1	5	3.8	4.2	1.1
T2	7	0.10	0.050	0.52
T2K	7, 8	0.23	0.52	2.3
KT3	8, 13	0.18	1.2	6.3
T3	13	5.5	16	2.9
T4	22	13	20	1.5
T6	27	0.59	0.57	0.97
KT8	39, 53	4.7	2.2	0.47
T8	53	3.5	0.91	0.26
T10	60	5.2	1.2	0.24
T11K	72, 73	1.6	1.6	1.0
T12	79	9.1	11	1.2
T13	86	14	23	1.7
T13K	86, 87	0.55	5.5	10
KT14	88	0.25	1.0	4.2
T15	99	4.7	6.3	1.3
KT16	100	0.27	0.31	1.2
T16		0.30	0.42	1.4

[a] HCC*$_{surface}$ samples were modified with 8 methyl groups per molecule by reaction with 10 mM formaldehyde. HCC*$_{solution}$ samples were modified with 8 methyl groups per molecule by reaction with 4 mM formaldehyde. Buffer: 4.4 mM potassium phosphate, pH 7.2. See Experimental Section for additional details of reaction parameters. [b] Tabular values shown are relative mass spectral intensities for HCC modified in solution; see Table 1, footnote c.

is in the solution phase. The presence of large amounts of the three peptides K*T3*T4, T3*T4, and T3*T4*T5 (see Table 3, column 3) demonstrates that K13 undergoes extensive modification in solution under these conditions. As a result, very little cleavage of K13 should occur in the digest of the solution control, which would severely limit production of T4*T5. The intensity arising from T4*T5 for HCC*$_{solution}$ would accordingly be artificially small, causing the intensity ratio for T4*T5 to be artificially large. This effect is readily understood by considering the hypothetical case in which 100% of K13 was methylated in the solution control. In that case, I_{T4*T5} for HCC*$_{solution}$ would be zero, and the intensity ratio for T4*T5 would become infinite. At only 1.5 methyl substituents per molecule, modification of K13 in the HCC*$_{solution}$ control is much less extensive as almost no K*T3*T4 or T3*T4*T5 were detected and the signal for T3*T4 was much weaker (see Table 3, column 6). A similar line of reasoning can be advanced for K*T8. This particular peptide carries K39, which would be well-exposed to the solution phase if binding occurs through the front face. The nearest lysines to either side, K27 and K53, both show high levels of modification in the HCC*$_{surface}$ samples, as evidenced by the presence of significant amounts of T6*T7 and K*T8*T9 (see Table 3, column 3). Accordingly, if the amount of K*T8 produced in a digest of HCC*$_{surface}$ became limited due to reduced cleavage at K27 and K53, the intensity arising from K*T8 for HCC*$_{surface}$ would become artificially small, as would the intensity ratio for K*T8. To the extent that this phenomenon does occur, the degree of protection exhibited by K39 will be overestimated.

Before more closely examining the methylated peptide data in Table 3, we will first evaluate whether the intensity pattern that arises from the detection of *unmodified peptides* is also consistent with front face binding. The data are shown in Table 4. For two sequential lysyl groups, K(n) and K(n+1), the $I_{surface}/I_{solution}$ ratio for the unmodified peptide containing K(n+1) will be high *only when both* lysines are well-protected in the bound state. On the other hand, a low intensity ratio will be favored when *either or both* of the sequential lysines experience little chemical protection. In Table 4, the intensity ratios for T2K, KT3, T3, T13K, and KT14 have the highest values; the ratios for T8 and T10 have the lowest. These results indicate that K8,

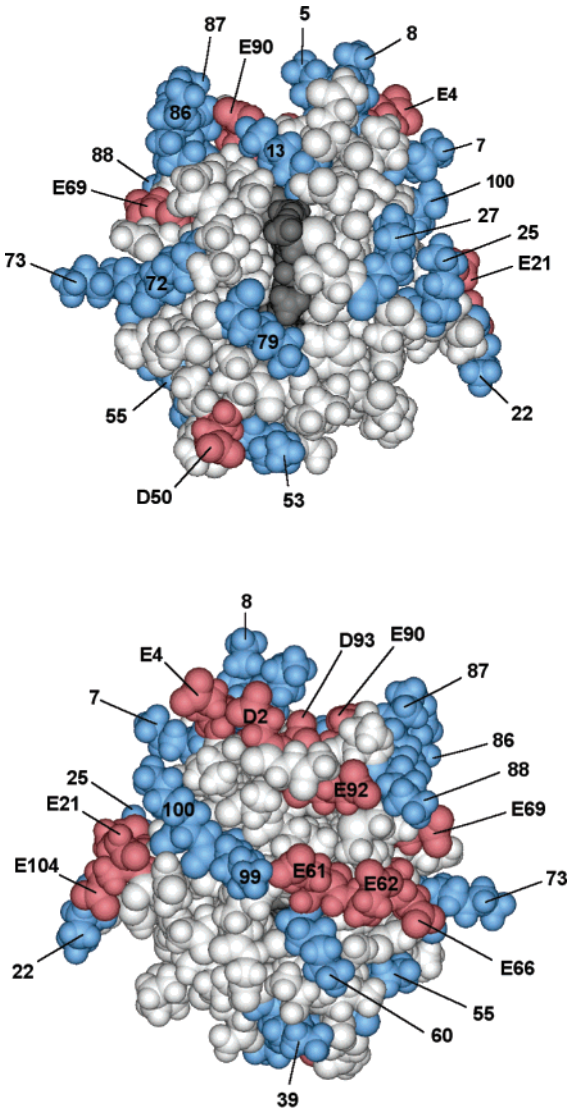

Figure 2. Conventional views of horse cytochrome *c*. Top: front face. Bottom: back face. Numbers without letter designation refer to lysines.

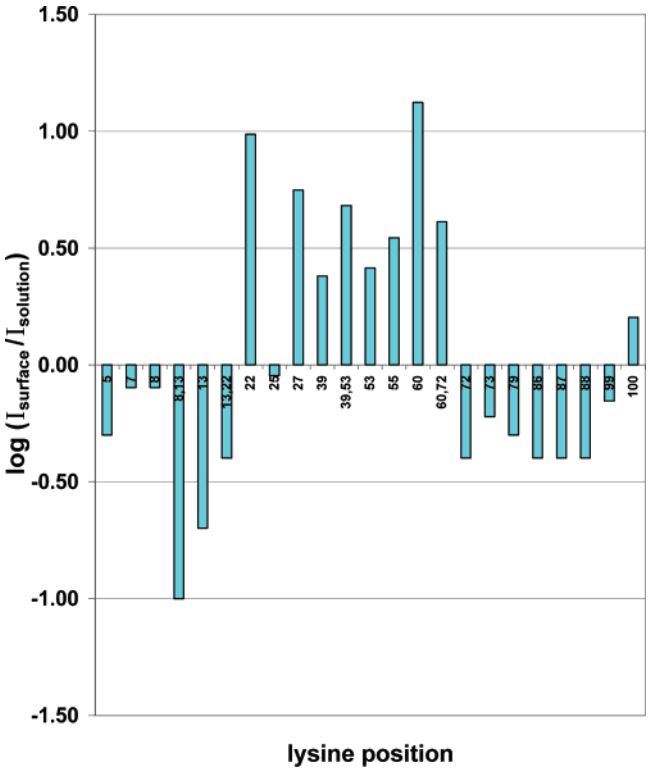

Figure 3. Reactivity of lysines in the adsorbed state relative to the solution state. Intensity ratio ($I_{surface}/I_{solution}$) taken from Table 3, column 5. Buffer: 4.4 mM potassium phosphate, pH 7.2.

Table 5. Ranking of Surface Regions of Cytochrome *c* with Regard to Chemical Protection Conferred by Adsorption. Lower numbers indicate better protection

		Relative Ranking[a]	
lysines	location[b]	Table 3	Table 4
5, 7, 8	upper right	4	n.r.[c]
13	top of heme edge	1	2
22	right edge	7	7
25, 27	right of heme edge	6	n.r.[c]
39, 53, 55, 60	lower back, Met-80 side	8	8
72, 73, 79	left front	3	n.r.[c]
86, 87, 88	upper left	2	1
99, 100	middle back, His-18 side	5	n.r.[c]

[a] Based on intensity ratio values, $I_{surface}/I_{solution}$, in Tables 3 and 4. [b] Refer to Figure 2. [c] Ranks 3−6 not resolvable from data in Table 4.

K13, K79, and K86−88 are well-protected, whereas K39, K53, and K60 are not, again implicating the front face in the binding interaction. We conclude that the distributions of modified (Table 3) and unmodified peptides (Table 4) are in good agreement. To elucidate a more precise binding orientation, however, analysis of methylated peptides will be the better approach to use because these data, for the most part, reflect modification at single lysine positions with the concomitant appearance of tryptic peptides featuring one missed cleavage. On the other hand, analysis of a data set for simple unmodified tryptic peptides is a strategy of inherently lower resolution because it depends on the reactivity of neighboring lysines (see above). Accordingly, only one representative set of unmodified peptide data has been presented in Table 4.

Binding Site and Orientation. To attempt a more precise analysis, we have found it convenient to subdivide the lysine population of HCC into "clusters" of 1−4 lysines based on proximity in the tertiary structure. These clusters are listed in Table 5. Using the intensity ratios in Table 3 for methylated peptides, these eight lysine clusters were ranked from lowest to highest reactivity in the adsorbed state (see column 3 in Table 5), which to a first approximation can be taken to represent their degree of exposure ranging from least to most. With this assumption, K13, lying just above the exposed heme edge, is the best protected, followed closely by the lysine cluster K86−88 found on the upper left region of the front face (see Figure 2). The three lysines stretching from the bottom of the exposed heme edge around the left side, K72/73/79, are also a well-protected region, although perhaps slightly less so than the first two. At the top of the protein just right of center is K5/7/8, a three-lysine cluster displaying moderate protection. On the back face, the lysine pair K99/100 is located mid-latitude on the His-18 side and ranks fifth in degree of chemical protection. The third and second most exposed regions of the molecule appear to be clusters K25/27 and K22, respectively, located to the right of the heme edge. Viewed conventionally (Figure 2), K22 demarcates the front and back faces of cytochrome *c* on the His-18 side and is further removed from K25 than is K25 from

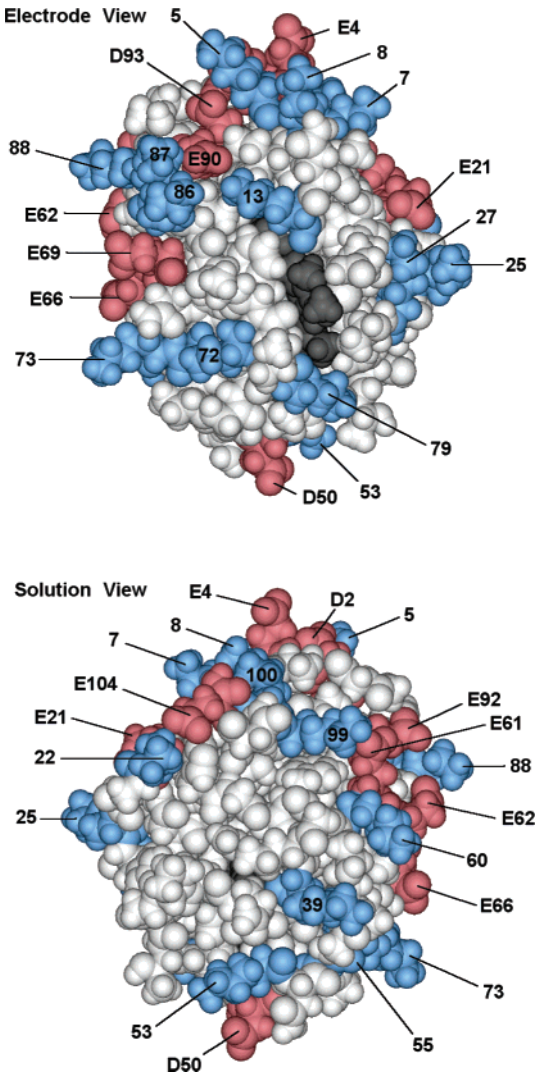

Figure 4. Proposed orientation of adsorbed horse cytochrome *c* on Au−$S(CH_2)_{10}COOH$ SAM at neutral pH. Top: view from the electrode surface. Bottom: view from the solution phase. Adsorption buffer: 4.4 mM potassium phosphate, pH 7.0, 10 mM ionic strength. Gold substrate: 10 μm diameter gold particles. Numbers without any letter designation refer to lysines.

K27, thus providing the justification to classify it separately. Finally, we identify the most exposed surface region as K39/53/55/60, a grouping located toward the bottom of the back face favoring the Met-80 side.

Collectively, the results in Tables 3−5 implicate the surface region of HCC lying above and to the left (Met-80 side) of the exposed heme edge. The proposed orientation is depicted in Figure 4 as views from the electrode surface and the solution phase. This orientation credits lysines 13, 86, 87, and 88 with being the least reactive sites followed closely by 72, 73, and 79. An inspection of the polypeptide backbone of HCC furthermore reveals that the α-carbons of six (13, 72, 73, 79, 86, and 87) of these seven lysines lie roughly in a plane. In Figure 4, the proposed orientation has this plane positioned approximately parallel to the substrate surface, effectively placing the α-carbons of these six lysines approximately equidistant from the SAM surface. This notion, albeit untested, implies that the energetic contributions of these six lysines to the lowest free energy binding state of HCC at the COOH SAM surface are similar. For the proposed orientation, the heme is tilted 35−40° relative to a surface normal.

As viewed from the solution phase (Figure 4B), the three clusters K22, K39/53/55/60, and K99/100 feature the most accessible lysines. These would also be the most reactive lysines if diffusional accessibility was the sole determinant of reactivity. The K39/53/55/60 grouping is, in fact, the most reactive region, followed by K22. Surprisingly, on the other hand, the K99/100 pair exhibits only average reactivity (Table 3), which we propose is a result of reactivity modulation by nearby aspartates and glutamates. Lysines 39, 53, 55, and 60 are found low on the back face in a region that is essentially devoid of carboxylates. On the other hand, K99 and K100 are imbedded in a concentrated region of negative charge provided by D2, E4, E61, E92, and E104. We postulate that HCC binding to the highly anionic COOH SAM fosters stronger electrostatic attraction between K99/100 and their backside carboxylate neighbors, resulting in an increase of pK_a and a decrease in reactivity for these two lysines. This modulation may occur due to the neutralization of positive charge on the upper front face from binding interactions, which would presumably remove a stabilizing electrostatic influence on the upper backside carboxylates. A direct field effect could also play an important role since K99/100 and the nearby carboxylates all lie within 5−15 Å of the COOH SAM surface for the orientation depicted in Figure 4. The possible modulation of lysine reactivity through lateral electrostatic interactions is an issue deserving of additional attention.

Interpretation of differential reactivity data, such as that presented in Tables 3 and 4, requires caution. A number of experimental issues can distort the data relative to a simplistic view that relative lysine reactivity in the adsorbed state reflects chemical protection in a structural sense. One example identified above was the modulation of lysine reactivity that can occur due to the effect(s) of interfacial electrostatic fields. Bosshard has provided a good description of other complications that can impact the interpretation of differential modification data.[26]

Comparison to Prior Investigations of Orientation. Several spectroscopic determinations of horse cytochrome *c* orientation on carboxylated organic surfaces have been reported.[12,16b,20] The most definitive determination was reported by Edmiston et al. for HCC adsorbed on arachidic acid LB films deposited atop waveguide-supported cadmium arachidate trilayers.[16b] A heme tilt angle of $\theta = 46 \pm 6°$ with respect to the surface normal was determined. By comparison, a tilt angle of $\theta \cong 35-40°$ at the Au/$S(CH_2)_{10}COOH$ interface was found in the present work (Figure 4). Considering the uncertainties, most notably the differences in film structure and the assumptions underlying the development of Figure 4, these two results are in good agreement. Other spectroscopic determinations of horse cytochrome *c* orientation on carboxylated surfaces were reported by Dick et al. for 6-mercaptohexanoic acid SAMs on silver-coated nanoparticles[20] and by Ataka and Heberle for 3-mercaptopropionic acid (3-MPA) modified gold films.[12b] In each case, cytochrome *c* was found to be positioned with its solvent-exposed heme edge side toward the substrate surface. In the latter study,[12b] a specific orientation for horse cytochrome *c* on 3-MPA was proposed that involved strong binding to lysines 13, 72, and 86, which is in excellent agreement with the orientation we are proposing for $C_{10}COOH$ SAMs. We also find

good agreement with recent nonspectroscopic results from Imabayashi et al., who measured electron-transfer rates for 12 monosubstituted CDNP lysine derivatives of horse cytochrome *c* adsorbed on 7-mercaptopentanoic acid SAMs.[8a] The most benign substitutions were found at positions 39, 60, and 99, which is in excellent agreement with Figure 4. Molecular simulations of horse cytochrome *c* adsorbed on Au(111)−$S(CH_2)_9COOH$ SAMs have recently been reported by Zhou et al. for different degrees of surface ionization.[40] Between 5 and 50% COOH ionization, horse cytochrome *c* was found to adsorb with the heme group nearly perpendicular to the SAM surface. In these simulations, lysines 25, 27, 72, and 79 were identified as having the strongest electrostatic interactions. Although there is qualitative agreement between these simulation results and our findings, significant differences do seem apparent.

Besides the carboxyl-terminated alkane class of adsorbents, notable investigations of horse cytochrome *c* orientation at two different anionic organic surfaces have been conducted.[16f,41] For HCC adsorption on a sulfonate-terminated silane-anchored C_{16} SAM, a heme tilt angle of 41 ± 11° was reported by Du and Saavedra using TIRF and linear dichroic absorption techniques.[16f] The second investigation, by Regnier's group, employed a novel chemical strategy, namely, thermally activated fixation of horse cytochrome *c* through covalent bond formation with lysine residues.[41] Although their work bears some resemblance to ours in that LC/MS of protein digests was the method used to determine lysine involvement in HCC binding interactions, two key differences stand out. First, the surfaces were chemically different. Xu et al.[41] investigated a unique anionic surface featuring succinimidyl sulfonate chemistry that was designed to covalently react with lysyl groups on dissolved HCC, thereby "trapping" the protein in the binding state. A second difference was the reaction time. Longer reaction times were required to covalently immobilize solution phase HCC molecules (up to 16 h) in contrast to the fairly short times required for reductive methylation of adsorbed HCC (5 min). Binding of HCC at the succinimidyl sulfonated resin was found to occur on the solvent-exposed heme edge side with approximately equal involvement of lysines 5, 7, 8, 13, 25, 27, 72, 73, 79, and 86−88. These findings are in qualitative agreement with the orientation depicted in Figure 4 and with recently published calculations from Yao and Lenhoff.[42]

Experimental evidence amassed over the past three decades points to a common surface domain on horse cytochrome *c* that dominates its binding to most negatively charged species and surfaces. This domain is located up and to the left of the solvent-exposed heme edge in the conventional front view of the molecule. This is the same region of the molecule that was identified in seminal studies by Bosshard,[23−26] Margoliash,[43−45] and Millett[46−49] of cytochrome *c* interaction with the reaction partners cytochrome oxidase, cytochrome *c* peroxidase, the cytochrome bc_1 complex, sulfite oxidase, and cytochrome b_5. Although the cytochrome *c* binding domain determined for cytochrome *c* was not identical for these five proteins, it always encompassed the exposed heme edge and clearly favored the upper left side of the front face. Lysines 8, 13, 25, 27, 72, 79, 86, and 87 were regularly implicated, with 13, 72, and 86 always strongly involved in binding interactions with all five reaction partners. This surface region of the protein also happens to contain the crossing point for the dipole axis. The involvement of the same approximate domain has now been identified in the present work in the low ionic strength binding of HCC to a $HS(CH_2)_{10}COOH$ SAM/gold surface. Considering the similarity of heme tilt angles, it seems reasonable to expect the same domain to be involved in binding to sulfonated SAMs[16f] and arachidic acid LB films.[16b] Moreover, Chang and co-workers[21] have reported a heme tilt angle of 41° for HCC adsorbed on hydrophilic fused silica, a finding that is consistent with the same specific domain being involved in binding to negatively charged inorganic surfaces.

Concluding Remarks

Employing a nonspectroscopic chemical method, we have determined the binding site on horse cytochrome involved in its adsorptive immobilization on a COOH-terminated self-assembled monolayer. The methodology is based on the differential chemical protection principle advanced by Bosshard in the 1970s for probing contact domains in protein−protein complexes. Further development of this methodology with respect to future cytochrome *c* studies is envisioned. The strategy itself is conceptually straightforward: minor total fractions of the lysyl residues in adsorbed horse cytochrome *c* are modified by reductive methylation, subsequently assayed by LC/MS analysis of tryptic digests, and compared to modified solution phase control samples. Because the use of trypsin is somewhat problematical, a future investigation of simpler digestion strategies would be clearly warranted. Trypsin is not very active at lysyl residues that have undergone modification. As a result, tryptic digests contain all the unmodified native peptides as well as many new peptides having one or more modified lysyl groups. Tryptic digests of modified HCC thus tend to be complex with ours containing some 2−3 times more detectable peptides than digests of native HCC. Moreover, the signal intensity of a given peptide is a function of multiple variables, including several of a chemical nature. Most importantly, intensity depends not only on the extent to which lysyl groups in the peptide itself are modified but also on the extent to which lysyl groups immediately before and after it in sequence are modified. These complications can largely be avoided by using a digestive enzyme that cleaves at amino acid residues other than lysine, such as Glu-C. The tradeoff, however, is the production of oligopeptides that feature multiple lysine residues irrespective of whether any have been modified or not. For example, one of the peptides produced by the digestion of HCC with Glu-C is expected to contain six lysine residues. To quantify the modification extent of individual lysyl groups in more lengthy peptides produced using this route will require the use of LC/MS/MS.

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The detection sensitivity of our instrumentation restricted the scope of this study with respect to surface area. Data reported in this paper were acquired for a typical sample size of 1 g of 10 μm diameter gold particles, which corresponds to a surface area of several hundred square centimeters. Going forward, this limitation on surface area can be greatly reduced by utilizing nanospray ESI LC/MS, which can improve the detection sensitivity on the order of 10^3 relative to conventional ESI LC/MS. With nanospray capability, determining protein orientation on planar surfaces on the order of 1 cm^2 should be feasible.

The universal nature of the methodology used should be noted. Although lysyl residues were exclusively modified in the present work, the generic approach is applicable to surface aspartates and glutamates, and thus most proteins would appear to be amenable to future investigation. In contrast to electronic spectroscopy, the methodology is not highly dependent on the nature of the substrate material or its surface geometry. Differential modification appears to be more universally applicable than previously described strategies that also do not rely on electronic spectroscopy. These include a priori protein modification with antibody or chemical probes,[8a,50−52] covalent fixation,[41] H/D exchange,[53] scanning probe microscopy,[54−57] and infrared spectroscopy.[12] Chemical strategies that involve a priori protein modification[8a,50−52] will always give rise to species that are no longer identical to the native protein. As a result, attachment of modifiers can, in general, be expected to alter the intrinsic binding properties of a given protein to a degree dependent upon the modifier structure and its location. The use of large modifiers, such as monoclonal antibodies,[50−52] will also impart significant limitations on the accuracy and precision that can be obtained for an orientation determination. Methods based on in situ covalent fixation,[41] although widely applicable to soluble proteins, are restricted to substrate surfaces derivatized with specific protein attachment chemistry. Another chemical strategy, H/D exchange,[53] requires that some protein denaturation occur as a result of the binding process. Imaging methods comprise a third major class of in situ strategies that complement spectroscopic and chemical methods. In situ tapping-mode AFM has been most successful in this regard,[54−57] but resolution has limited its appeal to large proteins of high aspect ratio or of otherwise distinguishable shape, such as antibodies and human transferrin. Finally, we note the recent development of an orientation-sensitive vibrational spectroscopic technique, surface-enhanced infrared difference absorption spectroscopy (SEIDAS).[12] To overcome the limited sensitivity of infrared spectroscopy, this technique relies on electrochemical modulation of the redox state of an immobilized protein, which precludes the use of nonredox proteins or nonconductive surfaces. On the other hand, the potential of SEIDAS for yielding new insights in regards to protein monolayer electrochemistry seems promising.

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Supporting Information Available: Modification reaction conditions; SAM compatibility; model compound reaction; mass spectrometry of holocytochrome *c*; LC/MS of tryptic digests of native and modified-in-solution-phase horse cytochrome *c*; predicted peptide frequency dependence on extent of modification. This material is available free of charge via the Internet at http://pubs.acs.org.

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