pH-Dependent Local Structure of Ferricytochrome c Studied by X-Ray Absorption Spectroscopy

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ABSTRACT We have studied, using x-ray absorption spectroscopy by synchrotron radiation, the native state of the horse heart cytochrome c (N), the HCl denatured state (U_1 at pH 2), the NaOH denatured state (U_2 at pH 12), the intermediate HCl induced state (A_1 at pH 0.5), and the intermediate NaCl induced state (A_2 at pH 2). Although many results concerning the native and denatured states of this protein have been published, a site-specific structure analysis of the denatured and intermediate solvent induced states has never been attempted before. Model systems and myoglobin in different states of coordination are compared with cytochrome c spectra to have insight into the protein site structure in our experimental conditions. New features are evidenced by our results: 1) x-ray absorption near edge structure (XANES) of the HCl intermediate state (A_1) presents typical structures of a pentacoordinate Fe(III) system, and 2) local site structures of the two intermediate states (A_1 and A_2) are different.

INTRODUCTION

This work deals with a study of the local structure of heme iron in ferricytochrome c (cyt-c) in acidic and basic solutions using x-ray absorption spectroscopy (XAS) at the iron K-edge. XAS is a short-range probe sensitive to variations in the local structure and its use has brought considerable contributions to the understanding of the detailed environment of specific atoms in biological systems (Durham et al., 1983; Bianconi et al., 1985). The characterization of conformational transitions and folding intermediates in proteins is important for the study of protein folding and the principles of structure stabilization. Horse cyt-c is a well-characterized globular protein both in the crystalline and solution state, and it represents a very useful model for protein folding studies (Scott and Mauk, 1996). The cyt-c has been shown to have three stable states in the acidic pH region, corresponding to native (N), unfolded (U_1), and compact intermediate or molten globule (A_1), with transitions between them (Goto et al., 1990a; Ohgushi and Wada, 1983; Fink et al., 1994). At the neutral pH (N state) cyt-c is a globular protein having two strong field protein ligands to the heme iron; an imidazole nitrogen of histidine 18 and a sulfur of methionine 80, coordinated in the axial position of the heme plane. When titrated with HCl in the absence of salt, the optical spectra of the protein show two transitions: initially unfolding in a single cooperative transition at around pH 3, resulting in a fully unfolded state, and at pH 2 (U_1 state), probably losing the heme axial ligands (Goto et al., 1990a; Babul and Stellwagen, 1972). However, the addition of more acid results in collapse to a compact intermediate or molten globule state, lacking the native methionine ligand (A_1 state pH 0.5) (Goto et al., 1990a).

The addition of NaCl to the protein in the U_1 state at pH 2 also leads to a collapse into a refolded intermediate state (Goto et al., 1990a, b), indicating that the chloride anion should play a key role in these acid- or salt-transitions to the molten globule state. This acid-salt transition is a phenomenon common to several quite different proteins. In the acid denaturation, intramolecular charge repulsion is the driving force for unfolding. The shielding of intramolecular charge-charge repulsion forces in the U_1 state by anion binding brings the formation of the intermediate one (Goto et al., 1990b). The A state has a significant amount of secondary structure, but substantially disordered tertiary structure. On the basis of structural and kinetic studies the molten globule state has been proposed as the major intermediate in protein folding (Goto et al., 1990b; Christensen and Pain, 1991; Ohgushi and Wada, 1983).

The nature of alkaline transitions observed on raising the pH to alkaline values (pH 9) has been extensively studied in recent years, especially in connection with the role of conformational changes in electron transfer. The bulk of spectroscopic evidence suggests that the alkaline form of the protein (the U_2 state) possesses a six-coordinate, low-spin heme iron that retains the native imidazole ligand of His-18, while the sulfur atom of Met-80 is replaced by another strong-field ligand, most likely a lysine amino group (Wilson and Greenwood, 1996).

Although several studies regard the nature of heme-binding groups in cyt-c under various native, basic, and acidic denaturing conditions, no final conclusions can be made yet. In fact, all information on the number and identity of axial ligands to the heme iron is indirect, mostly obtained from optical spectroscopy and absolutely not conclusive (Goto et al., 1990a; Scott and Mauk, 1996). The need for a more

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direct probe for studying the coordination and nature of the ligands is required. XAS technique is ideally suited to probe the local environment of iron atoms in cyt-c in dilute aqueous solution. Particularly, changes in the environment of the iron atom following conformational changes of the protein can be monitored, and thus information on the relationship between local and overall conformation can be obtained. In this work XAS at the iron K-edge was used to characterize the local environment of the heme iron in cyt-c in all the stable states N, U, and A of the protein, and to monitor the pH- and salt-induced transitions between them.

METHODS

Horse heart cytochrome c (type III) was obtained from Sigma Chemical Co. (St. Louis, MO). All absorption spectrophotometry was performed using the JASCO V-750 instrument; pH measurements and titrations were made using a Crison micro pH 2000 pH meter outfitted with microelectrodes. The concentration of native ferricytochrome was determined using an extinction coefficient of $1.06 \times 10^5\ M^{-1}\ cm^{-1}$ at 410 nm and 25°C (Babul and Stellwagen, 1972).

The experimental protocol was the following: 1) a pH titration was carried out on the protein from pH 7 to pH 12 using NaOH; 2) a pH titration was carried out on the protein from pH 7 to pH 0.5 using HCl in absence of salt; 3) a salt titration with NaCl was carried out over a 0–500 mM range, at the pH of maximum unfolding (at 60 mM HCl at about pH 2). To make comparison with folding studies based on other structural probes, the conformational transitions of cyt-c (at the concentration of protein used for XAS, 2 mM, which is a hundred times higher than those usually used for optical spectroscopy) were also studied by monitoring changes of the heme absorption spectrum. All the samples have been prepared from the same native solution by adding small amounts of highly concentrated acid-basic-salt buffers to reach the required molar ratio, consequently the added volumes did not change appreciably (within a few percent of the initial value) the protein concentration.

The x-ray absorption near edge structure (XANES) spectra of ferricytochrome c (2 mM of concentration) have been acquired at GILDA beamline (ESRF) at the iron K-edge at room temperature under low vacuum conditions in fluorescence geometry. Beam energies were defined using an Si[311] double crystal monochromator working in dynamical sagittal focusing mode. A seven-element ORTEC model IGLET-11150 × 7-S detector has been used. Calibration of the energy scale was achieved by measuring the fluorescence signal and the intensity transmitted out of a metal iron foil placed after the sample at the same time.

The acquisition statistics were fixed as follows: in the pre-edge zone (7080–7100) 1 s of integration time with a step resolution of 1 eV; in the XANES region (7100–7200) 5 s of integration time with 0.2 eV of step resolution. The fluorescence count jumped from ~60c/s/element before the edge (7100 eV) to ~600 c/s/element after the edge (7250 eV), giving a total count jump of ~21,000. The spectra have been normalized as described elsewhere (Congiu Castellano et al., 1989). The characteristic features of the six coordinated oxidized forms of cytochrome c are present (Labhardt and Yuen, 1979). Comparing the optical spectra of the native sample before and after the XANES acquisition, we deduced that no photoreduction occurred.

Model systems, such as hemin, and myoglobin (Mb) in different states of coordination have been compared with cyt-c spectra in order to have a first interpretation of our results. The XANES spectra of the model systems chosen in the present work were acquired at the D21 x-ray absorption beam line of LURE and have been published as part of previous independent studies (Della Longa et al., 1996, 1998; Boffi et al., 1999).

RESULTS

Optical absorption experiment

Acidification of a salt-free solution of cyt-c using HCl leads to an increase of the absorbance peak at 620 nm, characteristic of high-spin complexes. Fig. 1 shows the dependence on HCl concentration of the protein extinction coefficient at 620 nm for three different protein concentrations. A cooperative transition occurs at [HCl] = 14 mM for 0.8 mM concentration of protein, at [HCl] = 27 mM for 2 mM, and [HCl] = 65 mM for 5 mM concentration of protein, respectively.

Further addition of HCl leads to a second transition partially overlapping with the first. As shown in Fig. 1, the first transition appears to be resolved from the second one for low protein concentration only.

XANES experiment

In Fig. 2 an experimental XANES spectrum of native horse heart cyt-c (with features named P, B1, B2, C1, D, C2) and its derivative spectrum (with features named π, $\beta 1$, $\beta 2$, $\chi 1$, δ, $\chi 2$) are reported.

In Fig. 3 (*left panel*) the XANES spectrum of the native protein is compared with its denatured and intermediate states; in the right panel their differences are reported. The NaOH U_2 state (Fig. 3 *a*) spectrum shows variation on the intensity of the main peaks (C1, 7131 eV and D, 7137 eV), a decrease of the B1 (7121 eV) and B2 structure (7126 eV). The addition of HCl in the protein solution (U_1 state Fig. 3*b*, *b′*) still results in an increase of the intensity of the main peaks, while the C2 (7150 eV) structure disappears. At higher concentration of HCl (A_1 state, Fig. 3 *c*, *c′*) no new

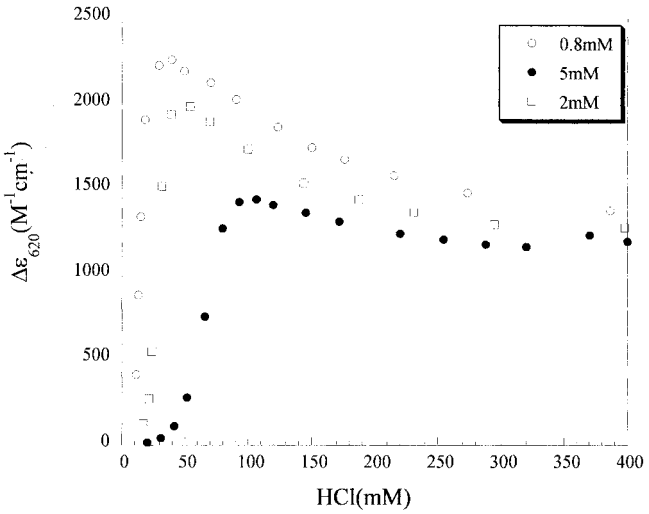


FIGURE 1 The effect of increasing concentration of HCl on extinction coefficient at 620 nm for three different cytochrome c concentrations is shown: (○) 0.8 mM; (□) 2 mM; (●) 5 mM. The size of the symbols is representative of the error.

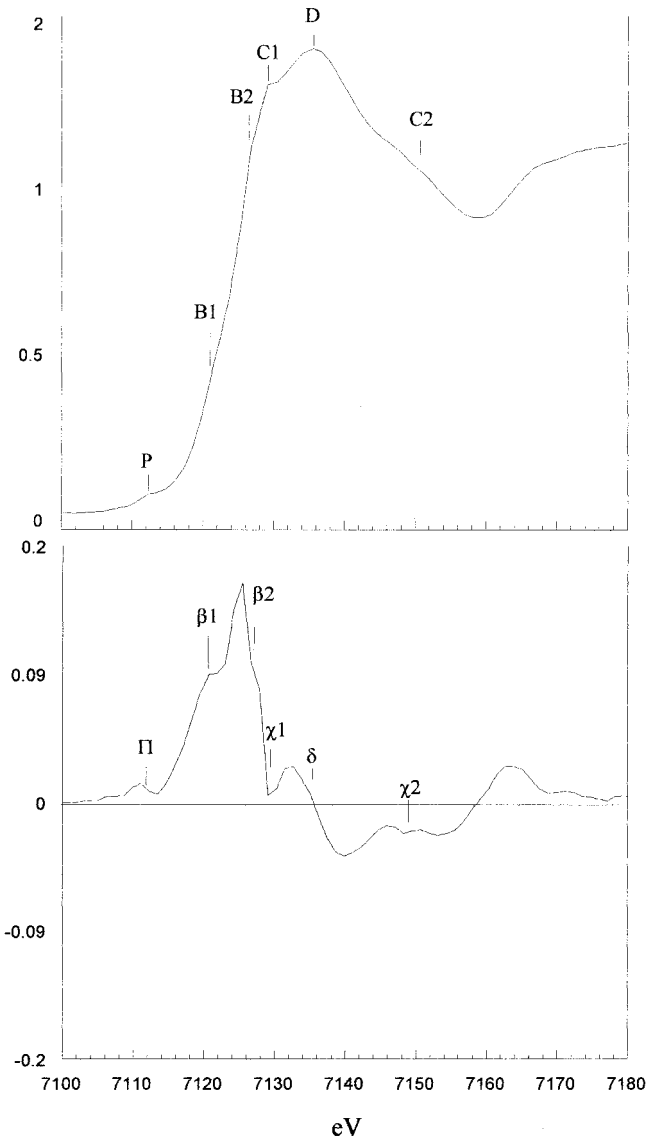


FIGURE 2 Experimental XANES spectrum of native horse heart ferricytochrome c (with features named P at 7111 eV, B1 at 7121 eV, B2 at 7126 eV, C1 at 7131 eV, D at 7137 eV, C2 at 7150 eV) and its derivative spectrum (with features named π, $\beta 1$, $\beta 2$, $\chi 1$, δ, $\chi 2$) is shown.

feature appears, while the main peak intensity decreases. The increase of the pre-edge peak P is also visible (Fig. 3 *c*, *inset*). The increase of peak P at 7111 eV and the overall shape of the XANES spectrum in the A_1 state is interpreted according to both theoretical arguments (Kutzler et al., 1980) and experimental evidence on heme model systems (Boffi et al., 1999) and heme proteins (Shiro et al., 1990), as indicative of a pentacoordinate iron-heme state.

The XANES spectrum in the presence of NaCl 0.4 M (A_2 state in Fig. 3, *d* and *d*′) exhibits some features similar to those of the native state: the C2 structure is again visible and the peak intensity seems to decrease toward the native state. The B1 structure is detectable. The structural differences (Fig. 3, *a*′, *b*′, *c*′, *d*′) are presented to evidence the structural changes respect to the native states, and are introduced as a comparison with model systems that will be discussed in the following section.

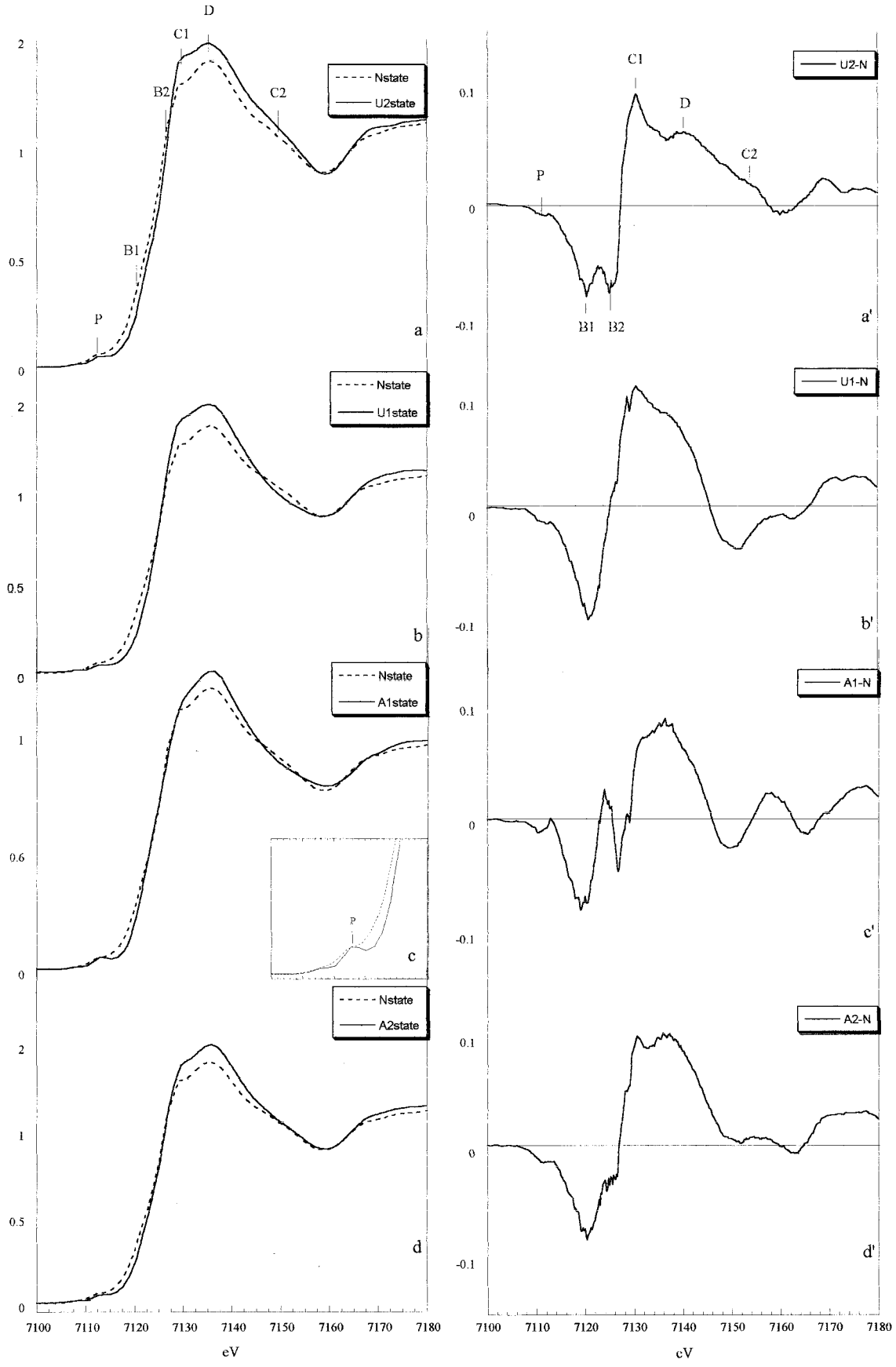
DISCUSSION

Optical spectra of cyt-c show the presence of two subsequent transitions at increasing HCl concentration (Fig. 1). The presence of two transitions, when titred in absence of salt, is a well-documented observation for this protein (Goto et al., 1990a; Fink et al., 1994). The first transition is ascribed to N-U and the second to U-A states. Our observations reveal an effect related to protein concentration.

As previously observed (Goto et al., 1990a; Babul and Stellwagen, 1972; Fink et al., 1994), HCl titration in the presence of NaCl can lead directly from native state to the A state, without the presence of an U state. The phenomenon seems due to the very strong efficacy of chloride, which induces a refolding: the effects of chloride, which should favor folding, should easily compensate the effect of protons, which favor unfolding. Our measurements show that the same effect appears at high protein concentration. In fact, as protein concentration increases, the N-U transition shifts to higher concentration of HCl (at pH 2 more HCl is needed for higher protein concentration) and it interferes with the U-A transition. Therefore the presence of Cl^- ions influences, even in this case, the transition upon HCl titration from N-U-A to N-A. This fact sets a limit to the concentration of protein to be used for XAS measurements for the characterization of the three states N, U, and A. In fact, at high protein concentration, upon decreasing the pH, the protein will go directly into the A state from the N state.

To clarify the coordination and the nature of the ligands to the heme in the different states induced by the addition of HCl, NaCl, and NaOH to the native cytochrome c, a comparison with model compounds has been carried on. The rationale of these comparisons lies in the fundamental assumption of the XANES signal as a local probe of the environment of the iron atom: the XANES spectra of two iron-heme systems having identical oxidation and spin state, and the same axial ligands should be nearly the same, little differences pertaining to small geometrical distortions or the presence of different ligands of the same nature (i.e., different amino acid).

In Fig. 4 a schematic representation of the model states that better represents our experimental spectra is shown. The hexacoordinated bis imidazole and the pentacoordinated mono-imidazole compounds (Fig. 4, *b* and *d*) reconstituted in SDS (sodium dodecyl sulfate) micelles have been characterized by XANES, optical absorption, and Raman spectroscopies (Boffi et al., 1999), while the Mb aquomet (Fig. 4 *c*) has been studied using XANES experimental and theoretical approach (Della Longa et al., 1998). The two hexacoordinate model compounds have been chosen to in-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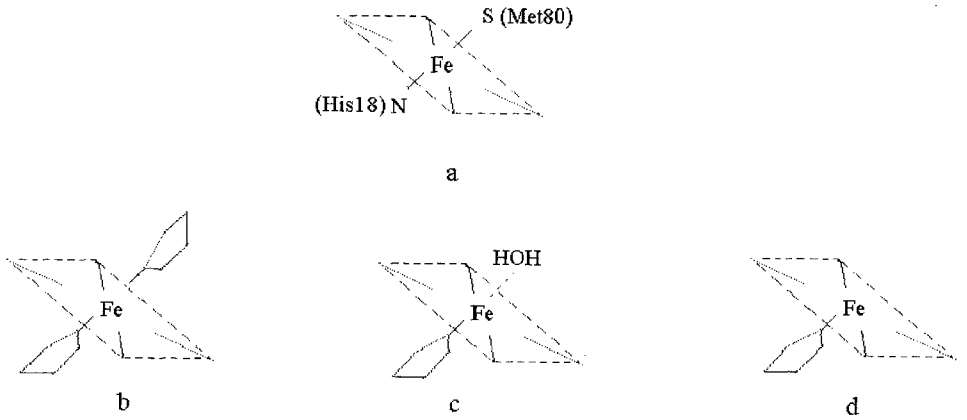


FIGURE 4 A schematic representation of the site structure for: (*a*) native cytochrome c; (*b*) bis imidazole hemin in SDS micelles (Boffi et al., 1999; (*c*) metmyoglobin at pH 4 (Della Longa et al., 1998); (*d*) 1,2-dimethyl-monoimidazole hemin in SDS micelles (Boffi et al., 1999).

terpret the two denaturant states U_1 and U_2, while the pentacoordinate system is shown to be the best model for the A_1 state.

In Fig. 5 the differences between the model compounds and the native state are compared with the differences of the spectra shown in Fig. 3. As it can be noted by a first look at Fig. 5, the spectral differences of the model compounds (*a*, *c*, and *e*) exhibit the same features of the spectral differences *b*, *d*, and *f*, respectively.

The NaOH (U_2) state shows the typical features of a bis-imidazole iron-heme adduct (Della Longa et al., 1998; Boffi et al., 1999). In Fig. 5 *b* it is possible to observe the goodness of the model chosen (a bis-histidine hemin, Boffi et al., 1999). The whole landscape of the experimental spectral differences is reproduced in the model (5 *a*); little difference in the relative intensity of the peaks is visible. The presence of a different amino-group bound to the iron-heme as sixth ligand cannot be excluded; a nitrogen of a lysine could be the second strong ligand, as suggested by Wilson and Greenwood (1996).

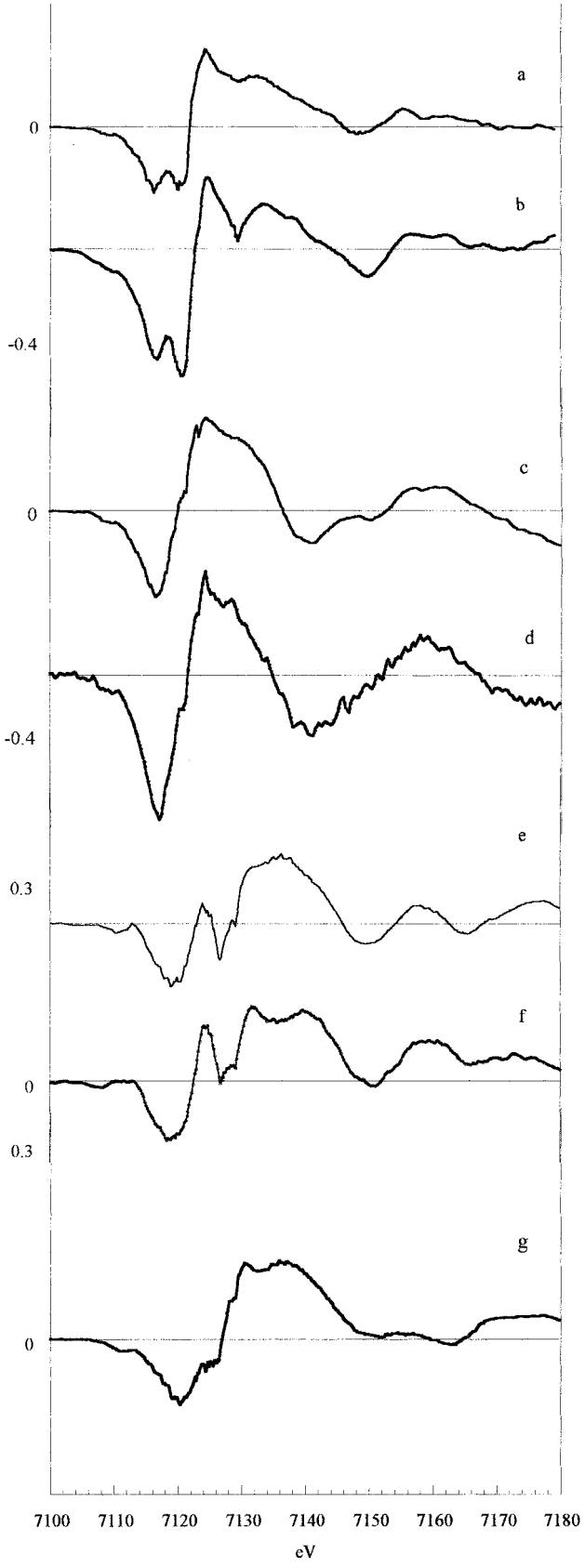
In Fig. 5, *c* and *d* the same comparison with model and experimental spectra is presented for the HCl denaturant (U_1) state. The XANES spectrum presents characteristics of an Mb-Met at low pH aquomet (Della Longa et al., 1998). Even in this case the landscape of the spectral differences for the experimental is reproduced for the model (5 *c*). Small differences between the model and our spectrum could be due to the presence of further components having a different sixth ligand: that is a histidine in the model.

The interpretation for the two intermediate states has been approached in the same way. For what concerns the A_1 (HCl induced) intermediate state (Fig. 5, *e* and *f*), a pentacoordinate hemin with an imidazole as fifth ligand reconstituted in SDS micelles (Boffi et al., 1999), represents the best model state. The differences between the model and the native spectra and between A_1 experimental and the native spectra are even more similar with respect to the others (Fig. 5, *e* and *f*). In particular, in Fig. 3 the lower intensity of the peak B_1 at 7120 eV is visible for the A_1 state; this is due, according to multiple scattering theory (Durham, 1988), to a lower number of collinear multiple scattering pathways with respect to octahedral complexes. Moreover, the pre-edge peak P at 7111 eV is enhanced in pentacoordinate systems. In octahedral, centrosymmetric clusters, very small pre-edge peaks are referred to as $A_{1g}T_{2g}$ or $A_{1g}E_g$ dipole forbidden transitions (Kutzler et al., 1980). For non-centrosymmetric clusters, the peaks increase in intensity due to metal p-d orbital mixing. Other mechanisms have been reported to contribute to intensity, e.g., metal 3d orbital mixing, magnetic dipole and electric quadrupole transitions, and vibronic coupling with the metal-ligand vibrations (Sano et al., 1992). Even if the main mechanisms are not yet fully understood, the pre-edge peak is always observed to be enhanced in pentacoordinate with respect to hexacoordinate hemeproteins (Oyanagi et al., 1987; Shiro et al., 1990; Ikeda-Saido et al., 1992).

The presence of a histidine coordinated in our model suggests that the native histidine (His-18) could be bound to the heme in the A_1 state, in agreement with previously reported work (Babul and Stellwagen, 1972).

According to Dyson and Beattie (1982) the intermediate state, characterized by optical and CD spectroscopies, is a mixture of a high-spin form and a low-spin form, both having His-18 coordinated to the iron, while Met-80 is coordinated to the iron in the low-spin form only. Goto et al. (1990) suggest that this intermediate state (state II in Dyson and Beattie) is the A_1 state: moreover, they show that the two intermediate states A_1 and A_2 have similar features.

FIGURE 3 *Left panel:* All the experimental spectra are shown: (*a*) the native oxidized form of the cytochrome c spectrum (N) is compared with its NaOH denatured form (U2; pH 12); (*b*) the native oxidized form of the cytochrome c spectrum is compared with its HCl denatured form (U1; pH 2); (*c*) the native oxidized form of the cytochrome c spectrum is compared with its HCl denatured form (A_1; pH 0.5); (*d*) the native oxidized form of the cytochrome c spectrum is compared with the HCl/NaCl titrated form (A_2). *Right panel:* The spectral differences of the spectra shown in the left panel are shown.



Our data are not in agreement with these hypotheses. In particular, the clear increase of the P peak in A_1 is not explained by supposing the presence of native iron-heme form (Met-80 coordinated). Our evidences strongly suggest a pentacoordination for the A_1 state. Moreover, our results show great differences between the A_1 and A_2 states at local structure levels. In fact, the A_2 state (Fig. 3 *d*) presents some features of the native compounds; i.e., the C2 peak at 7150 eV is reproduced (the difference between the N and the A_2 state at 7150 eV is close to 0, as shown in Figs. 3 *d′* and 5 *g*). However, a valid model for such systems is not available, leaving uncertainties about the characterization of this state.

CONCLUSIONS

New features appear in this experiment. First, we can exclude the presence of a mixture of hexacoordinated (Met-80) and pentacoordinated iron-heme adducts for the A_1 state at equilibrium. A second important feature is relative to the A_2 state. A valid model for this is not yet available. However, our results suggest the presence of species different from the A_1 state. This consideration leads us to conclude that the acid-salt induced refolding is not related to the coordination at the heme pocket level.

Regarding the U_1 state the absence of Met-80 in the model is in agreement with previous literature being not well defined the nature of the sixth ligand. The assignment of the NaOH denaturant state (U_2) as a bis-histidine species is not in contrast with the bulk of spectroscopic evidence (Scott and Mauk, 1996), founding another strong ligand in the substitution of the Met-80. To definitively characterize the nature of the sixth ligand for these two cases and for the A_2 state, XANES simulations will be executed and EXAFS studies could be attempted to measure the ligand distances and geometry (Meneghini and Morante, 1998).

In this work the comparison of XANES protein spectra with experimental model systems has been proved to be a powerful approach for the characterization and interpretation of XANES data. In the absence of a definitive theoretical approach this seems to be the only way to interpret a XANES spectrum. Our results not only partially clarify the uncertainties on the coordination and on the nature of the ligands at the active site level of cyt-c, but as a consequence give new opportunities for the interpretation of the acid-salt induced intermediate states.

FIGURE 5 A comparison between the spectral differences shown in Fig. 3 and spectral differences between experimental and experimental model compound spectra: (*a*) bisimidazole hemin–native cyt-c; (*b*) NaOH denatured state–native cyt-c; (*c*) metmyoglobin at pH 4–native cyt-c; (*d*) HCl denaturant state–native cyt-c; (*e*) 1,2-dimethyl imidazole hemin–native cyt-c; (*f*) HCl intermediate state–native cyt-c; (*g*) intermediate NaCl intermediate state–native cyt-c.

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