Absorption and Raman Spectroscopy Study of Cyt c – Thiol Complexes in Acidic Solutions

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Abstract. Absorption UV-VIS and pre-resonance Raman spectra of acidic cyt c solutions with a series of thiols added (thiophenol, n-propanethiol, isopropanethiol, L-cysteine, dithiothreitol, 2-mercaptoethanol, N-acetyl-L-cysteine, p-acetamidothiophenol, 2-mercaptoethanamine, thioglycolic acid and mercaptopropionic acid), are presented. Interactions of cyt c molecule with the thiols were studied with the aim to identify binding of the thiols with the cyt c heme as its iron axial ligands. Absorption and Raman spectra showed some correlation between maxima of 700 nm region absorption band (typical for Fe-S axial bond in cyt c heme) and also wavenumbers of spin state marker and axial ligand sensitive Raman bands on one, and pK_a constant values of appropriate thiols on the other hand. These results imply thiol replacement of Met-80 from axial bond with heme iron and suggest that the force of Fe-L-cysteine axial bond is very close to the native axial bond (Fe-Met) for cyt c in neutral solution.

Key words: Ferricytochrome c — Heme axial ligand — Thiol — Raman scattering — Absorption spectroscopy

Introduction

Cytochrome c (cyt c) is hemoprotein which acts as an electron carrier in the mitochondrial respiratory chain. Its prosthetic group, heme, is covalently attached to the polypeptide chain through thioether linkages with two cysteine residues, Cys-14 and Cys-17 (Salemme 1977). Heme iron binds axially with two other aminoacid residues, His-18 and Met-80, which keep the porphyrin ring in planar conformation and form a low-spin heme complex (Spiro and Loehr 1975).

Recent studies of cyt c interactions in solution with its redox partners in res-

piratory chain, cytochrome oxidase and cytochrome reductase (Smith et al 1981, Mauk et al 1982, Michel and Bosshard 1984, Michel et al 1989) have shown certain conformational changes of cyt c apoprotein to be associated with these interactions, resulting in a weakening of the iron-methionine axial bond and in low-to-high spin state transition of heme iron (Hildebrandt and Stockburger 1990) The conformation of low spin heme is planar whereas the high spin porphyrin has a pyramidal conformation due to stronger axial ligand drawing out of the iron atom from the heme plane (Rakshit and Spiro 1974, Spiro and Loehr 1975) The extent of the iron atom being drawn out from the heme plane depends on the force of its bond with the axial ligand (Rakshit and Spiro 1974) Interactions of cyt c with negatively charged interfaces of biological macromolecules (cytochrome oxidase and cytochrome reductase), electrodes or polyanions (Chottard et al 1987, Hildebrandt and Stockburger 1986, 1989a, 1989b, 1990) showed that, in dependence on experimental conditions, cyt c heme may exist in various spin states due to altered force of the Fe-Met axial bond

To characterize the force of this bond at individual possible spin and conformation states of heme the Fe-Met (i e Fe-S) bond was modified by cyt c methionine oxidation (Myer et al 1987) or by replacing Met-80 at axial position of heme by some exogenous thiols or thioethers (Oshio et al 1985, Bayer et al 1969, Sadeque et al 1987, Schejter and Plotkin 1988, Antalik and Bagelova 1986) Binding with cyt c heme of a thiol series differing from each other in nucleophilicity can be expected to provide information about the native Fe-S axial bond (Fe-Met-80) in cyt c molecule It is due to the nucleophilicity or the electron density on sulphur atom of thiols can be simply represent by pK_a a constants (Friedman 1973)

Electrostatic interactions of thiol anions (at low concentrations) with the charged surface of cyt c molecule in neutral solution lead to heme reduction (Ginsburgh and Everse 1978), no immediate thiol interactions with cyt c heme can be observed In the concentration range as used in the present study ($10^0 - 10^{-2}$ mol l^{-1}), thiols can interact not only with the cyt c surface but also with its chromophore (heme) group (Sadeque et al 1987, Schejter and Plotkin 1988) Also, low pH of solution (below 3) provides weakening of electrostatic interactions with the protein surface, since in similar conditions thiols are present as undissociated molecules (see values of pK_a constants, Table 2) So direct thiol interactions with cyt c heme can be studied in these conditions

The visible absorption spectrum of cyt c contains a band at 695 nm typical of Fe-Met axial bond in cyt c (Schechter and Saludjian 1967) A band around 700 nm also appears in spectra of cyt c with sulphur exogenous ligands (Schechter and Saludjian 1967) and is typical for Fe-S axial bond not only in cyt c but also in spectra of other hemoproteins (Bayer et al 1969) The band at 730 nm was observed in spectrum of cyt c with 2-mercaptoethanol axially bound to heme (Antalik and Bagelova 1986) The wavelength of this band depends on the heme spin

state (Smith and Williams 1968).

Raman scattering is a method very sensitive to heme conformational changes (low-to-high spin transition) and to axial ligand exchanges in hemoproteins. Cyt c Raman bands at 1502, 1582 and 1636 cm^{-1} (spin state markers) (Spiro and Strekas 1974) are assigned to the inner bonds vibrations of their porphyrin rings (Abe and Kitagawa 1978). Stronger frequency reductions of these bands are associated with a change in the spin state of heme iron (from low- to high-spin) (Spiro and Strekas 1974), i.e. a change of the porphyrin ring conformation from planar to pyramidal. The extent of the iron atom being pushed out from the heme plane depends on the force of iron axial-ligand bond (Rakshit and Spiro 1974). Thus the spin state markers can be used to identify binding of some exogenous ligands with various force constants of Fe-ligand bond. Other Raman bands (1375 and 1564 cm^{-1} for cyt c (Spiro and Strekas 1974) are sensitive to the nature of iron-ligand bond (Abe and Kitagawa 1978).

Raman and absorption spectra of cyt c in acidic solutions with a series of thiols added, presented in this paper, have indicated substitution of Met-80 as a second axial ligand by the thiols. The spectra show a correlation between maximum wavelengths of the "700 nm region" absorption band or Raman spin state markers and axial ligand sensitive bands of cyt c complexes with the respective thiols and pK_a constants of the thiols, i.e. their nucleophilicity.

Materials and Methods

Cyt c (horse heart, Sigma, Type III) was purchased and used without further purification. Neutral (aqueous) and acidic (0.1 mol.l^{-1} Gly/HCl buffer, pH 2.6 for Raman, and pH 3 for absorption experiments) cyt c solutions were used. Heme concentrations were 10 $^{-4}$ mol.l^{-1} for Raman and 2.5.10^{-5} mol.l^{-1} for absorption measurements. Experiments were carried out at 25 °C.

Thiols: thiophenol; n-propanethiol; isopropanethiol (all Aldrich); 2-mercaptoethanol (Sigma); L-cysteine (Calbiochem); dithiothreitol (Serva); N-acetyl-L-cysteine; p-acetamidothiophenol (Koch-Light-Laboratories), 2-mercaptoethaneamine; thioglycolic acid; and mercaptopropionic acid (all Lachema) (concentration range 10^0–10^{-2} mol.l^{-1}) were added to acidic solutions of cyt c used to eliminate the high ability of thiols to reduce cyt c heme. For Raman measurements (taking about 15–20 minutes), a lower pH value of buffer (2.6) was necessary. Values of pK_a constants of thiols (with the exception of p-acetamidothiophenol) have been taken from a paper by Danehy and Parameswaran (1968). The pK_a constant of p-acetamidothiophenol (6.1) was measured by the spectrophotometric method at 0.025 mol.l^{-1} 4-morpholineethanesulphonic acid (MES, Serva). The range of pK_a of thiols used in this study in this study was 6–11.

Absorption spectra were obtained using a Shimadzu UV 3000 spectrophotometer. Samples were measured in 1 cm pathlength cells.

Raman spectra were obtained with 514.5 nm excitation (Ar$^+$ laser, 80 mW power at the sample) by using computer controlled Jobin-Yvon HRD 1 double monochromator (holographical gratings – 1200 gr/mm). A rotating glass cell of 3 ml inner volume was

used. A cooled Hamamatsu R585 photomultiplier, working in photoncounting regime, was
used for detection. Spectral data were recorded at 2 cm^{-1} intervals with 1 s integration
time and a spectral slit width of 10 cm^{-1}. Repeated (6–10 times) recordings were taken
and usual methods of computer data processing were employed (background and buffer
contribution substraction, smoothing by FFT, etc. (Miškovský and Mojzes 1989)).
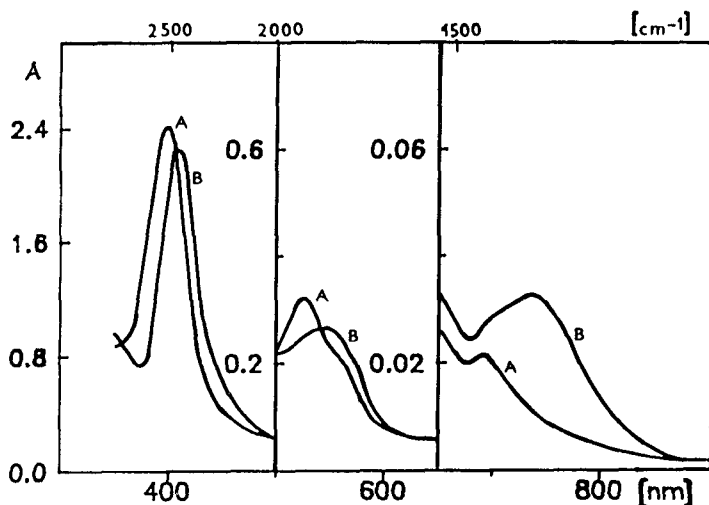

Figure 1. Absorption spectra of cyt c ($2.5.10^{-5} \text{ mol.l}^{-1}$) in acidic solution (0.1 mol.l^{-1}
Gly/HCl buffer, pH 3) (A) and in acidic solution with 2-mercaptoethanol (0.8 mol.l^{-1})
(B).

Results and Discussion

Absorption spectra analysis

The complexes of oxidized cyt c with thiols give electron absorption spectra dif-
fering from those of native cyt c (Fig. 1). While small changes of band maxima
caused by the thiol presence in cyt c solution appear in Soret (around 400 nm)
and α, β (above 500 nm) regions (as reported by Oshio et al. 1985), significant
changes concern the 700 nm spectral region. The intensity of 695 nm band, char-
acteristic for Fe-Met axial bond (Schechter and Saludjian 1967), decreases and a
new band appears shifted to the red region (Fig. 1). The maximum value of the
"700 nm region" absorption band (up- or down-shifted in comparison to 695 nm)
depends on the kind of the thiol derivative added (Tab. 2). The range of pK_a
values of the thiol derivatives series used has been wide (6–11), suggesting that

the derivatives chosen appreciably differ from each other in nucleophilicity of their sulphur atoms (Friedman 1973). The shifts range of maxima values exceeds 100 nm (2350 cm^{-1} on wavenumber scale in this spectral region) (Tab. 2), and the values of wavenumber maxima are roughly proportional to pK_a constant values of the respective thiol derivatives (Fig. 2). The results obtained suggest a lower energy of electron transition in cyt c-thiol complexes for thiols with higher pK_a.
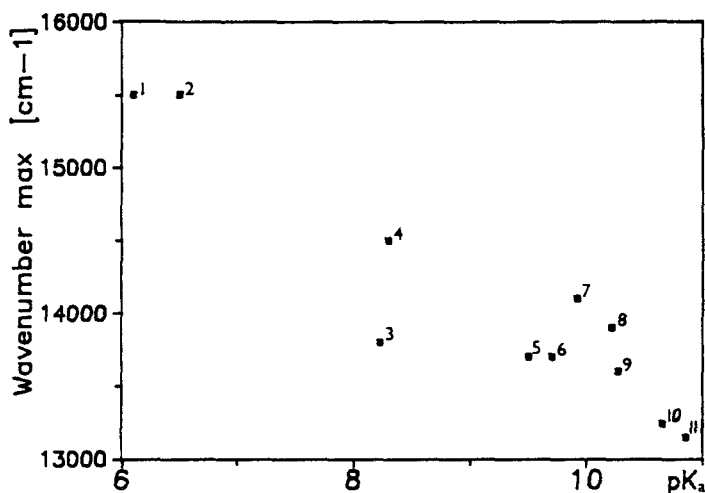
**Figure 2.** The dependence of the wavenumber of the "700-nm absorption band" maxima of cyt c – thiol complexes on pK_a constant. Wavenumber values and thiols, see Tab. 2; pK_a constants from Danehy and Parameswaran (1968). For the conditions see legend to Fig. 1.

The 700 nm spectral region is sensitive to high or low spin heme iron state in hemoproteins (Smith and Williams 1968). Iron spin changes are accompanied by structural changes of porphyrin ring which are not detectable by spectrophotometry. Raman scattering was used to obtain more detailed information about thiols binding to cyt c molecule (to confirm axial ligand replacement by the thiol derivatives added). A series of five thiols (derivatives 1, 4, 5, 8 and 9, Tab.2) was chosen for Raman measurements.

Raman spectra analysis of acidic cyt c solution

Raman spectra were obtained for acidic cyt c solutions with five thiol derivatives added. A 514.5 nm laser line was used, i.e. Raman spectra were excited in pre-resonance region of β heme absorption band. It implies partial enhancement of A_{2g}, B_{1g} and B_{2g} symmetry modes (Spiro and Loehr 1975), but also A_{1g} vibration

modes, which are enhanced with Soret region excitation (Spiro and Loehr 1975), are rather intensive.
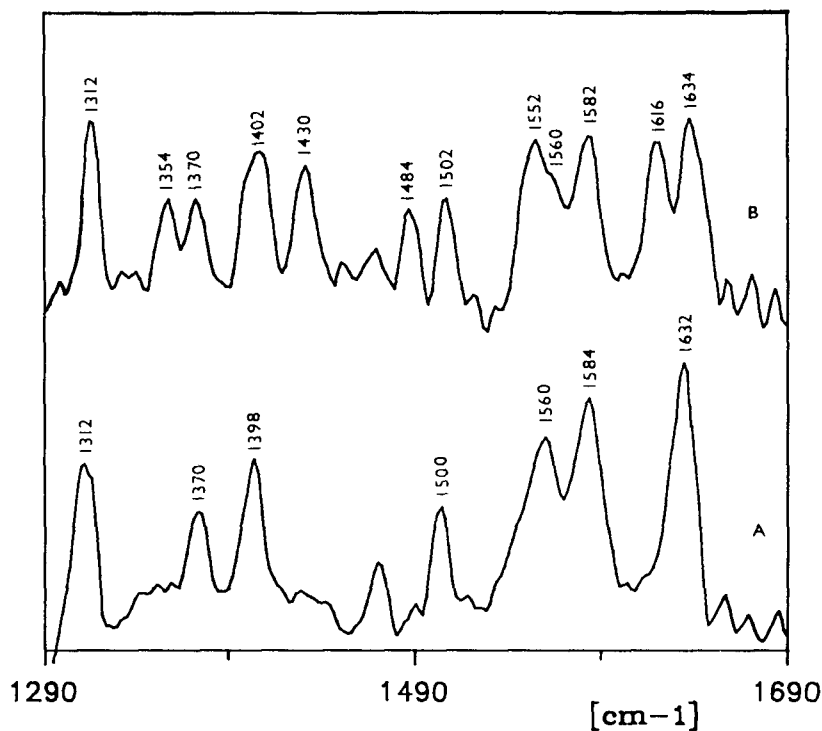
Figure 3. Pre-resonance Raman spectra of neutral (*A*) and acidic (*B*) cyt c solutions. Excitation at 514.5 nm.

In comparison to Fig. 3*A* (cyt c at neutral pH, "neutral form"), the spectrum in Fig. 3*B* (cyt c at pH 2.6, "acidic form") shows some changes of spin state markers and axial ligand sensitive bands. These changes suggest an exogenous ligand (buffer anion) replacement of Met-80 from sixth binding place of iron atom, as also observed by Stellwagen and Babul (1975) and a change of porphyrin ring conformation from planar to pyramidal, caused by different force constants of Met and substituting exogenous ligand.

A common feature of all bands sensitive to changes from planar to pyramidal conformation or to molecular kind of heme iron axial ligand in "acidic form" of cyt c (Fig. 3*B*) in comparison to its "neutral form" (Fig. 3*A*), is a relative intensity decrease of bands typical of "neutral form" and the appearance of new bands shifted apart. In agreement with Spiro and Strekas (1974), Abe and Kitagawa

(1978), and Hildebrandt and Stockburger (1986) these new bands are assigned to the same normal vibrations as the original bands correspond to, but for the cyt c molecules with replaced heme axial ligand by buffer anion. This replacement caused a planar-to-pyramidal conformation change of porphyrin ring. The decrease of "neutral form" bands intensity is a result of a reduced numl r of native ligated heme molecules (Met-80 remained as axial ligand of heme iron). In particular,

Table 1. Spin state markers and axial ligand sensitive Raman bands of cyt c molecule. Wavenumbers of "neutral" and "acidic form" are from Fig. 3.

Assignment[1]	Band position $[cm^{-1}]$	
	"neutral form"	"acidic form"
ν_{10},symmetry B_{1g} spin state marker F $\nu(C_\alpha C_m)$, $\nu(C_\alpha C_6)$	1632	1634
		$1616^{(2)}$
ν_{19}, symmetry A_{2g} spin state marker C $\nu(C_\alpha C_m)$, $\nu(C_\alpha C_6)$	1584	1582
ν_{11}, symmetry B_{1g} ax. ligand sens. band $\nu(C_6 C_6)$, $\nu(C_6\text{-perif.subst.})$	1560	1560
		$1552^{(2)}$
ν_3, symmetry A_{1g} spin state marker E $\nu(C_\alpha C_m)$, $\nu(C_\alpha C_6)$	1500	1502
		$1484^{(2)}$
ν_4, symmetry A_{1g} ax. ligand sens. band $\nu(C_\alpha N)$, $\delta(C_\alpha C_m)$	1370	1370
		$1354^{(2)}$

(1) – assignment of bands to the respective porphyrin normal vibration, and characterization of symmetry and main bonds contributions to these normal vibrations are according to Abe and Kitagawa (1978)

(2) – contribution of the respective "acidic form" cyt c vibration.

1. Band 1632 cm^{-1} for "neutral form" (Fig. 3A), designated F spin state marker and corresponding to ν_{10} normal vibration (see Tab. 1) (Abe and Kitagawa 1978) appears at 1634 cm^{-1} "acidic form" (Fig. 2B) with a decreased intensity (it corresponds to molecules with native axial ligands of heme). The new band appearing at 1616 cm^{-1} for "acidic form" corresponds to ν_{10} vibration of ligand exchanged heme molecules (strong-to-weak ligand exchange) (Spiro and Strekas 1974; Abe and Kitagawa 1978; Myer et al. 1987).

2. The intensity of the band at 1582 cm^{-1} – C spin state marker, ν_{19} heme normal vibration (see Tab.1) – was observed to decrease for the "acidic form"

(Fig. 3B) in comparison to the "neutral form" (Fig. 3A). The intensity increase in 1550–60 cm^{-1} region and the appearance of a new band at 1552 cm $^{-1}$ the spectrum of the "acidic form" are assigned to the superposition of ν_{19} vibration in heme molecules with exchanged axial ligand (down-shift) and ν_{11} vibration in both ("neutral" and "acidic") heme forms. The band at 1560 cm^{-1} in Fig. 3A is assigned to ν_{11} normal vibration (see Tab. 1) and is sensitive to molecular species of iron axial ligands (Abe and Kitagawa 1978). Thus, contribution of this vibration in "acidic form" heme molecules may be present at 1552 cm^{-1} (Fig. 3B).

3. Raman bands at 1500 cm^{-1} ("neutral form" spectrum, Fig. 3A) and 1502 cm^{-1} ("acidic form" spectrum, Fig. 3B) are E spin state markers assigned to ν_3 heme normal vibration (see Tab. 1) in "neutral form" (planar porphyrin ring conformation) heme molecules. The Raman band at 1484 cm^{-1} ("acidic form" spectrum, Fig. 3B) is assigned to ν_3 vibration in "acidic form" (ligand exchanged, pyramidal conformation of porphyrin ring) heme molecules (Spiro and Strekas 1974; Abe and Kitagawa 1978; Hildebrandt and Stockburger 1986, 1989a, 1989b; Myer et al. 1987).

4. The 1370 cm^{-1} band in "neutral form" spectra (Fig. 3A) corresponds to ν_4 heme normal vibration (see Tab. 1), and is very sensitive to molecular species of axial ligand (Abe and Kitagawa 1978). "Acidic form" spectrum (Fig. 3B) in comparison to the former one shows a splitting of the 1370 cm^{-1} band into two bands (1354 and 1370 cm^{-1}). The new band (1354 cm^{-1}) is assigned to ν_4 vibration in "acidic form" heme molecules.

Spin state markers and axial ligand sensitive Raman band frequencies of "neutral" and "acidic form" cyt c are listed in Tab. 1.

In comparison to Fig. 3A, there is another interesting change in "acidic form" spectrum (Fig. 3B) which is the appearance of the 1430 cm^{-1} band near the 1402 cm^{-1} band. The band at 1402 cm^{-1} corresponds to ν_{29} heme normal vibration (symmetry B$_{2g}$) with major contributions of C$_\alpha$C$_\beta$ and C$_\beta$-per.subst. bonds stretching vibrations (Abe and Kitagawa 1978). This band is not directly sensitive to axial ligand exchange. The interpretation of the 1430 cm^{-1} band is uneasy: it may also correspond to ν_{29} heme vibration due to the influence of buffer on porphyrin via surrounding apoprotein. This band appeared in all spectra measured in acidic solutions in the presence of thiols (Fig. 5B–F).

The intensive band at 1312 cm^{-1} corresponds to ν_{21} normal vibration (symmetry A$_{2g}$) with major contribution of outer heme parts: bending of C$_m$H and stretching of C$_\alpha$C$_\beta$ bonds (Abe and Kitagawa 1978), which are not directly sensitive to axial ligand exchange. The band does not change in intensity or wavenumber, either in pH 2.6 cyt c solution or in the presence of any of the thiols tested, and was used as an internal standard.
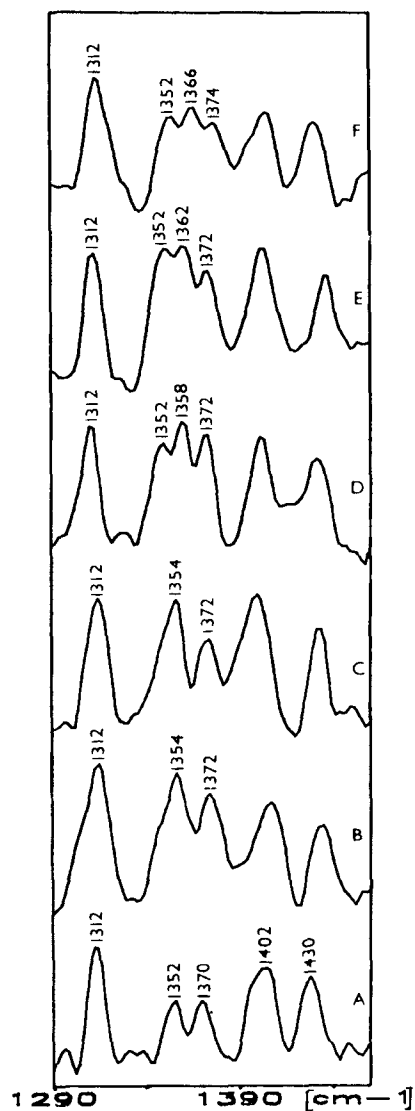
Figure 4. Pre-resonance Raman spectra of acidic cyt c solutions: cyt c alone (A), and in the presence of der.1 (B), der.4 (C), der.5 (D), der.8 (E) and der.9 (F); 1540—1690 cm^{-1} region. Excitation at 514.5 nm. For designation of thiols see Tab. 2.
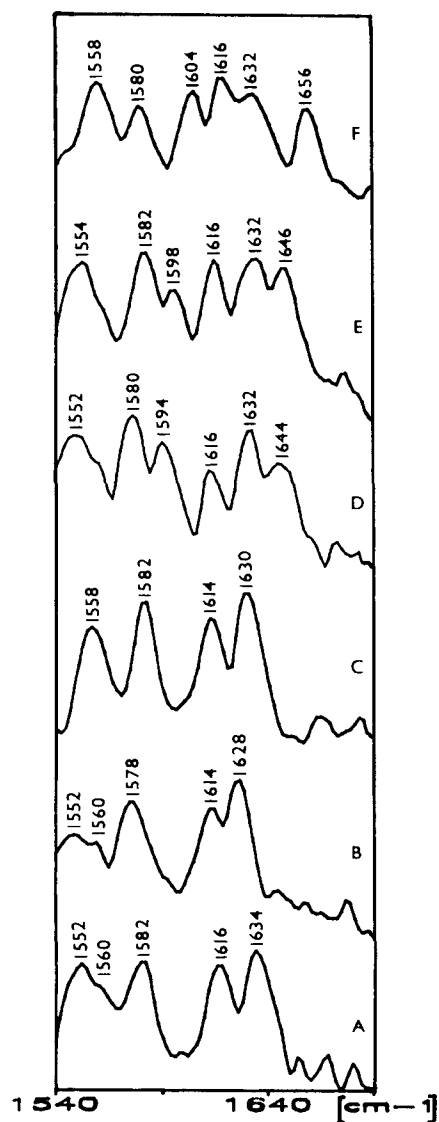
Identification of thiols as iron axial ligands in cyt c molecules

A common feature of Raman spectra of acidic cyt c solutions with one of the five various thiols added (derivative 1, 4, 5, 8 and 9, Tab. 2) (Fig. 4B–F, Fig. 5B–F) in comparison to that of acidic cyt c solution (Fig. 4A, Fig. 5A) is the appearance of new bands in spin state markers and axial ligand sensitive heme bands regions. The spectra in Fig. 4B–F and Fig. 5 B–F are superpositions of Raman scattering

Figure 5. Pre-resonance Raman spectra of acidic cyt c solutions: cyt c alone (A), and in the presence of der.1 (B), der.4 (C), der.5 (D), der. 8 (E) and der.9 (F); 1290—1460 cm^{-1} region. Excitation at 514.5 nm. For designation of thiols see Tab. 2.

contributions from native ligated (Met–80 as second axial ligand), "acidic form" (buffer anion as axial ligand) and "thiol binding form" (thiol as axial ligand) heme molecules. Raman bands assigned to heme vibrations in cyt c molecules with the respective thiols axially bound suggest heme conformation closer to planar (low-spin), typical for cyt c in neutral solution (Spiro and Loehr 1975), than to pyramidal (high-spin) conformation of "acidic form" cyt c molecules with exogenous buffer ion axially bound. This tendency in spectra agrees with what has been reported by

Table 2. Typical values of pK_a constants for the thiol series and absorption and Raman scattering data for cyt c binding with the thiols.

No.	Derivative	$pK_a^{(1)}$	$\lambda_{max}[nm]^{(2)}$ $(\nu_{max}[cm^{-1}])$	Raman bands$[cm^{-1}]^{(3)}$		
				ν_{10}	ν_{19}	ν_4
1	p-acetamidothiophenol CH_3-CONH-C_6H_5-SH	6.1	645 (15,500)	1628	1578	1354
2	thiophenol C_6H_5-SH	6.5	645 (15,500)			
3	2-mercaptoethaneamine NH_2-CH_2-CH_2-SH	8.23	725 (13,800)			
4	L-cysteine COOH-CH(NH_2)-CH_2-SH	8.3	690 (14,500)	1630	1582	1354
5	dithiothreitol SH-CH_2-$(CH(OH))_2$-CH_2-SH	9.5	730 (13,700)	1644	1594	1358
6	N-acetyl-L-cysteine COOH-CH(NH-CO-CH_3)CH_2-SH	9.52	709 (14,100)			
7	2-mercaptoethanol OH-CH_2-CH_2-SH	9.7	730 (13,700)			
8	thioglycolic acid COOH-CH_2-SH	10.22	719 (13,900)	1646	1598	1362
9	mercaptopropionic acid COOH-CH_2-CH_2-SH	10.27	735 (13,600)	1656	1604	1366
10	n-propanethiol n-CH_3-CH_2-SH	10.65	755 (13,240)			
11	isopropanethiol CH_3-CH(CH_2)-SH	10.86	760 (13,150)			

(1) – according to Danehy and Parameswaran (1968)
(2) – wavenumbers of "700 nm region" absorption band for cyt c – thiol complexes
(3) – spin state markers (ν_{10}, ν_{19}) and axial ligand sensitive band (ν_4), wavenumbers from Figs. 4, 5.

Bayer et al. (1969) and is due to force constant values of Fe-S bonds for particular thiols (values closer to Fe-Met than to that of the bond force constant of Fe-buffer anion) as will be discussed below.

1. Region 1610 – 1660 cm^{-1}

In all Raman spectra of cyt c with thiols, bands at 1616 cm^{-1} and 1632 cm^{-1} are assigned to ν_{10} normal vibration in "acidic" and "neutral form" heme molecules,

respectively. Contributions of "thiol binding form" heme molecules of ν_{10} vibration are present for bands at 1628, 1630, 1644, 1646 and 1656 cm^{-1} for thiol derivatives 1, 4, 5, 8, 9 (Fig. 4B–F), respectively. Bands at 1628 and 1630 cm^{-1} (derivatives 1 and 4, respectively) are superpositions of "neutral" and "thiol binding form" contributions of ν_{10} normal vibration. In comparison to 1632 cm^{-1} (Met-80 axially bound), these wavenumber values suggest that derivatives 1 and 4 are weaker ligands than Met (down-shifted bands), whereas the other derivatives tested draw the iron atom even more to the heme plane (up-shifted bands) than does native ligand (Spiro and Strekas 1974; Abe and Kitagawa 1978).

2. Region 1550 – 1610 cm^{-1}

Some interesting bands were observed in 1550–1610 cm^{-1} region: at 1594, 1598 and 1604 cm^{-1} for derivatives 5, 8 and 9 (Fig. 4D–F), respectively. They are assigned to ν_{19} normal vibration in cyt c molecules in which the corresponding thiols are axially bound. Up-shifts of these band wavenumbers in comparison to the "neutral form" value (1582 cm^{-1}) indicate that they more strongly draw heme iron into the heme plane (Spiro and Strekas 1974; Abe and Kitagawa 1978).

The contributions of the "thiol binding form" heme molecules for spectra with derivative 1 and 4 (Fig. 4B,C) appear at 1578 and 1582 cm^{-1}, respectively, together with the "neutral form" contributions of ν_{19} normal vibration.

The shape of the 1552 cm^{-1} band with a shoulder at 1560 cm^{-1} in the "acidic form" spectrum (assigned to superposition of ν_{11} normal vibration, axial ligand sensitive (Abe and Kitagawa 1978) in "acidic" and "neutral form" of heme, and to ν_{19} vibration of "acidic form" heme molecules) changes if thiols are added (Fig. 4B–F). A general feature of these changes is an intensity decrease of the 1552 cm^{-1} band and an intensity increase of the shoulder at 1560 cm^{-1} in cyt c spectra with thiols in comparison to the "acidic form" spectrum. This phenomenon also indicates backtransition of "thiol binding form" heme to a conformation similar to that of the "neutral form" heme (Fig. 3A).

3. Region 1350 – 1370 cm^{-1}

An interesting phenomenon in "thiol binding form" spectra (Fig.5D–F) is the appearance of some new bands at 1358, 1362 and 1366 cm^{-1} for derivatives 5, 8 and 9 spectra, respectively. They are assigned to ν_4 heme normal vibration. The wavenumbers of the new bands depend again on the type of the binding thiol. Bands characteristic for "neutral" and "acidic form" heme molecules and corresponding to ν_4 normal vibration (axial ligand species sensitive) are at 1370 cm^{-1} and 1352 cm^{-1}, respectively. In derivatives 1 and 4 spectra (Fig. 5B,C), the "thiol binding form" contributions appear at 1354 cm^{-1} together with "acidic form" contributions of ν_4 heme normal vibration (indicating weak axial ligand bond of derivative 1 and 4).

The analysis of Raman spectra of cyt c in the 1300–1700 cm^{-1} region confirmed the results obtained from absorption measurements (Tab. 2): in experimental conditions used (strong acidic buffer solution), the thiols series used can bind with heme iron atom and replace native axial ligand (or buffer anion). Due to the strong reducing effect of the thiols on cyt c heme it was impossible to increase the concentrations of the thiols in solution. As a result, the spectra presented are superpositions of several bands corresponding to the same vibrations of the three heme states present in solution: "neutral" , "acidic" and "thiol binding form".

The observed up- and down-shifts of Raman bands corresponding to ν_{10} and ν_{19} vibrations (spin state markers) and to ν_4 vibration (axial ligand sensitive) for thiol derivatives 1, 4, 5, 8 and 9, are in good correlation with changes observed in the 700 nm absorption region and also with differences of pK_a constants between the individual thiols (Tab. 2). Down-shifts of these three bands for derivatives 1 and 4 spectra indicate that the two derivatives are weaker axial ligands of heme iron than native ligand, Met-80, in neutral solution (derivative 4 is close to Met-80), while the spectra of the other thiols tested (up-shifted bands) indicate that they are stronger ligands than is Met-80 (they draw the iron atom to the heme plane more strongly than does native ligand).

The present absorption and Raman spectra analysis suggest that the force of axial bond with L-cysteine (derivative 4) bound with heme iron is very close to the value of Fe-Met bond for cyt c in neutral solution, i.e. to the value for iron bond with native cyt c axial ligand.

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