Volume 281, number 1,2, 205–208 FEBS 09592 April 1991

ADONIS 001457939100302V

Spectroscopic characterization of the copper(I)-thiolate cluster in the DNA-binding domain of yeast ACE1 transcription factor

Jose R. Casas-Finet[1], Stella Hu[1], Dean Hamer[2] and Richard L. Karpel[1]

[1]*Department of Chemistry and Biochemistry, University of Maryland Baltimore County, Baltimore, MD 21228, USA* and [2]*Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA*

Received 7 November 1990; revised version received 11 February 1990

A polypeptide containing the amino-terminal region of ACE1 (residues 1–122; 122*), the activator of yeast Cu-metallothionein gene transcription, shows charge-transfer and metal-centered UV absorption bands, and orange luminescence which are characteristic of Cu-cysteinyl thiolate cluster structures. These spectral features are abolished by the Cu(I) complexing agents CN^- and diethyldithiocarbamate or exposure to acid, but not by the Cu(II) chelator, EDTA. Binding of the polypeptide to its specific DNA recognition site, but not to calf-thymus double-stranded DNA, induces quenching of its Tyr and Cu-S cluster luminescence emission. The CD spectrum is characteristic of a tightly folded structure that may be organized around the Cu cluster.

DNA-binding protein; Metal ion; Luminescent complex

1. INTRODUCTION

The ACE1 protein of *Saccharomyces cerevisiae* is responsible for induction of the yeast metallothionein gene *CUP1* in the presence of Cu [1] and Ag ions [2]. The amino-terminal half of the 24 kDa ACE1 protein contains all 12 Cys residues, an excess of basic residues, and binds specifically to a *CUP1* upstream activator sequence (termed UAS_C) in the presence, but not in the absence, of Cu(I) or Ag(I) [2]. The carboxy-terminal portion of ACE1 is not required for DNA binding in vitro, contains no cysteine residues, is highly acidic, and is involved in transcriptional activation in vivo and in vitro [2,3]. In this article, we characterize the spectral properties of residues 1–122 of ACE1, a polypeptide termed ACE1(122*), which carries a protease-resistant domain capable of binding Cu(I) and DNA in vitro, and examine the effect of these ligands on its spectral features.

2. MATERIALS AND METHODS

ACE1(122*) was overproduced from an expression plasmid constructed by site-directed mutagenesis according to [4]. A *Nco*I site was introduced at the translation initiation site of the ACE1 gene [2]. A *Nco*I-*Bgl*II fragment was subcloned into the T7 expression plasmid Pet3D cleaved with *Nco*I and *Bam*HI. The resulting PNB plasmid encodes the first 122 amino acids of ACE1 together with the carboxy-terminal 19 amino acid sequence ADPAANKARKEAELAAATAEQ derived from the vector. This plasmid was introduced into BL11 (DES) pLysS for expression [5], a F^- OmpT rB^- mB^- *E. coli* carrying a lambda lysogen DES (with immunity of region of phage 21, lac I, lacUV5 promoter, and T7 polymerase) and the chloramphenicol resistance plasmid pLysS. Cells were grown at 37°C for 3 h, then induced for 2.5 h with 0.4 mM IPTG in the presence of 1 mM $CuSO_4$.

The expressed Cu-ACE1(122*) protein was purified by sonication (3 × 30 s) of resuspended frozen cells, followed by ultracentrifugation (100 000 × *g*, 45 min). The supernatant was subjected to heat treatment at 70°C for 7 min in 10 ml aliquots, and a heavy white precipitate was removed by centrifugation at 100 000 × *g* for 10 min. The resulting ice-cold supernatant was subjected to a 20%/50% saturated ammonium sulfate cut. The precipitate was resuspended in 20 mM HEPES, pH 8.0, containing 10% glycerol, 1 mM 2-mercaptoethanol and 1 mM PMSF (Buffer I), and run onto a 2 ml heparin-Sepharose column equilibrated with Buffer I containing 0.1 M KCl. The column was washed with 4 ml aliquots of the equilibration buffer with additional KCl in 0.1 M increments up to 0.8 M, and 2 ml fractions were collected. Cu-ACE1(122*) eluted in the 0.4–0.6 M KCl fractions. These fractions were pooled, diluted to 0.2 M KCl with Buffer I, and run onto a 1 ml CM-Sepharose column equilibrated with Buffer I containing 0.2 M KCl. Elution was carried out by 2 ml steps of 5 mM potassium phosphate buffer, pH 6.8 (Buffer II) containing 0.3–0.6 M KCl in 0.1 M increments; Cu-ACE1(122*) eluted at 0.4 M KCl. Peak fractions were chromatographed on Sephadex G25 in Buffer II, and concentrated by ultrafiltration on Centricon 10 or CentriCell 60 devices (10 kDa M_r cutoff). The protein was >98% pure as assayed by Coomassie brilliant blue-stained 15% SDS-polyacrylamide gel electrophoresis, and appeared as a single peak in elution profiles on a reverse-phase C_{18} μ-Bondapak HPLC column. High concentration samples required an ionic strength above 50 mM KCl to avoid aggregation.

Cu-ACE1(122*) samples were kept anaerobically under N_2 at −70°C in sealed containers until used. Protein samples were prepared in either 5 mM potassium phosphate, pH 6.8, or in 10 mM sodium cacodylate, pH 6.15, buffers. Buffers were degassed and saturated with nitrogen; sample handling was done in a glove bag under nitrogen atmosphere. Protein concentration was obtained by amino acid analysis, which revealed a composition identical to that predicted from the nucleotide sequence; metal content was determined by atomic absorption spectroscopy. The Cu-ACE1(122*) samples used

Correspondence address: J.R. Casas-Finet, Department of Chemistry and Biochemistry, University of Maryland Baltimore County, Baltimore, MD 21228, USA

for this work contained molar Cu:polypeptide ratios of 6.8 ± 0.2. ACE1(122*) samples were functionally saturated with copper, since addition of Cu-acetonitrile to the final preparations did not result in an increase of DNA binding activity.

The UASeL oligonucleotide is an annealed 22 bp sequence containing a high affinity binding site with the following sequence [5,6]:

(5' GATGCGTCTTTTCCGCTGAACC 3')
(3' CTACGCAGAAAAGGCGACTTGG 5')

Absorption spectra were acquired at 25.0 ± 0.1°C with a Gilford Response spectrophotometer using matched 1-cm pathlength Suprasil quartz cells with teflon stoppers. Luminescence studies were carried out on a SLM 48000S spectrofluorimeter equipped with double monochromators containing holographic gratings with 1500 grooves per mm. Excitation was performed with a 450 W Xe arc lamp. Detection was accomplished with cooled red-sensitive photomultiplier tubes offset to null the dark counts. Spectra were collected in the ratio mode using as a quantum counter a triangular quartz cell containing 3 $g \cdot l^{-1}$ of rhodamine B solution. Emission spectra were collected with a Glan-Thompson polarizing filter inserted in the vertical position in the emission path, in order to prevent an artifactual drop in sample emission intensity around 630 nm which is due to anomalous reflection off the holographic grating. As this significantly reduces the signal intensity, measurements at fixed wavelength were carried out without this correction. Excitation monochomator slits were set at 1 nm bandwidth, where sample photobleaching was undetectable. Emission monochromator slits were set to the appropriate resolution (usually at 2 or 4 nm bandwidth). Samples were held in a dual pathlength (0.2 × 1.0 cm) Suprasil quartz cuvette in a thermostated cell-holder, with the narrow pathlength facing the excitation beam. 200 μl samples were used in a capped cell; the sample compartment was maintained under nitrogen atmosphere. Data acquisition was performed by averaring 16 readings per wavelength. Data were processed by an IBM-PC/XT computer using software supplied by SLM.

Quantum yield determinations were carried out using as a standard aqueous tris(bipyridyl)ruthenium(II) chloride (absorbance matched at 305 nm), whose luminescence quantum yield (0.042 ± 0.003 at 25°C) [7] is independent of excitation wavelength from 280 to 560 nm [8].

Circular dichroism spectra were collected at 25.0 ± 0.1°C on a AVIV 60DS CD spectrometer over the wavelength range 200–320 nm, using strain-free Suprasil quartz cells with 0.1 or 0.2 cm pathlength. Five spectra were averaged to reduce the noise level; buffer blanks were run for each set of CD spectra and subtracted from the raw data. Smoothing was performed by fitting the data with a third degree polynomial over a sliding ten-point window.

3. RESULTS AND DISCUSSION

Fig. 1 shows the absorption spectrum of Cu-ACE1(122*), which exhibits a maximum in the far UV (due to n–π^* electronic transitions in the polypeptide backbone) and a broad transition characterized by a shoulder around 265–270 nm (which contains the overlapping contributions of π–π^* electronic transitions of aromatic side-chains and the charge-transfer absorption of the multicopper(I)-thiolate clusters). A fourth derivative of the absorption spectrum (not shown) exhibits discrete peaks at 207 and 273 nm, the latter suggesting the presence of Tyr chromophores, as expected from the primary sequence of Cu-ACE1(122*). Tyrosine absorption is more clearly revealed by the spectrum obtained at pH < 1, which results in displacement of Cu by H^+ ions and exhibits an absorption maximum at 274 nm (Fig. 1). A difference absorption spectrum shows that the copper cluster absorbance is broad (centered around 260 nm), weak (with a molar absorption coefficient of approximately 2100 $M^{-1} \cdot cm^{-1}$ at 260 nm) and extends down to the visible region of the spectrum (not shown). The cluster absorption is the dominant component of the spectrum at 250 nm, and can be used to monitor Cu(I) displacement from the protein by hydrogen ion competition, a process with half-maximal absorbance at pH 0.9 (Fig. 1, inset).

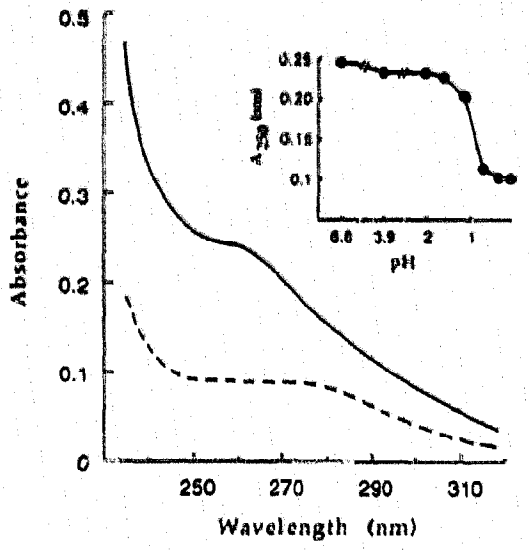

Fig. 1. UV absorption spectrum of ACE1(122*) in 5 mM potassium phosphate, pH 6.8 (solid line), and after acidification to pH 0.5 with HCl (dashed line). A titration curve displaying the absorbance at 250 nm vs. pH is shown in the inset.

Cu-ACE1(122*) exhibits a characteristic orange luminescence at room temperature with excitation light

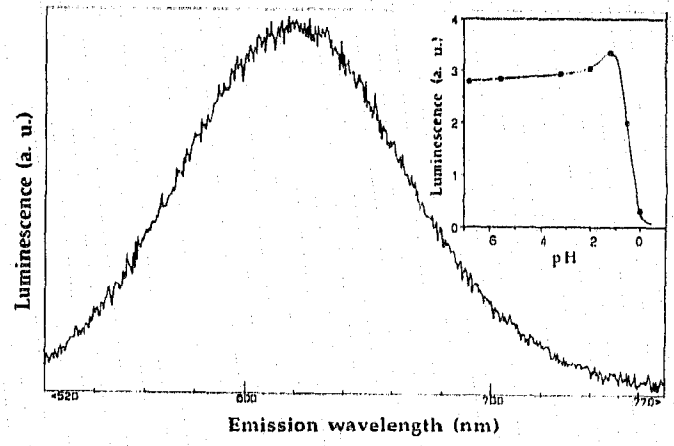

Fig. 2. Luminiscence emission spectrum (uncorrected) of ACE1(122*) (10 mM) in 5 mM potassium phosphate, pH 6.8, at 25°C. Excitation wavelength was 270 nm (1 nm bandpass), and the emission monochromator was set at 8 nm resolution; other conditions were as described in section 2. Inset: titration curve obtained monitoring the emission intensity at 610 nm vs. sample pH, adjusted with HCl aliquots.

ranging from the far UV to the visible; uncorrected emission was maximal at 619 nm (Fig. 2). The large Stokes shift is characteristic of Cu(I)thiolate clusters. The shape of the emission spectrum is approximately gaussian, with a slight skew to its blue side; its linewidth (FWHM) of 2800 cm^{-1} is almost identical to that of yeast metallothionein Cu-S cluster [9]. The excitation spectrum exhibits two maxima at 253 and 325 nm (Fig. 3). Since the emission spectrum is independent of the excitation wavelength, this suggests the existence of either: (i) several excited singlet states (resulting in partially overlapping electronic transitions) which cascade down to the singlet state with lowest energy before decaying to the ground state; (ii) an electronic transition to a singlet state possessing partially resolved vibronic bands; or (ii) as in the previous cases but with intersystem crossing to a triplet state from which the luminescence originates. The quantum yield of luminescence was 0.013 ± 0.001 at 25°C, which is about twice that of native Cu-metallothionein [9].

The luminiscence of the Cu(I)-thiolate cluster was affected by aerobic incubation, which resulted in a 20% emission loss but marginal decrease in absorbance at 250 nm upon exposure to air over several hours (not shown). The luminescence intensity but not λ_{max} varied with temperature, with the quantum yield rising by 1.70 ± 0.01% per degree with decreasing temperature over the range investigated (7–37°C). If linear with temperature, this would result in ca. 4-fold enhancement at liquid nitrogen temperature (77K). Luminescence emission intensity was unaffected by acidification of the sample in the pH range from 6.8 to 2, as was the wavelength of maximum emission. Further acidification to lower pH values led to a reduction in emission yield as Cu(I) dissociated from ACE1(122*) (Fig. 2, inset), with a titration mid-point at pH ~ 0.5, in good agreement with that obtained by monitoring the concomitant reduction in A_{250}. Luminescence emission was also inhibited by addition of the Cu(I) chelating agents potassium cyanide or diethyldithiocarbamic acid but not by the Cu(II) chelator, EDTA (not shown). Denaturation of Cu-ACE1(122*) with 6 M GuHCl abolished the Cu-S cluster luminescence (not shown).

The effect of the cis-acting control sequences of the CUP1 promoter on the luminescence emission of the Cu-ACE1(122*) peptide was investigated using the UAS_cL oligonucleotide, a double-stranded 22mer containing a single copy of the upstream activator sequence. Equilibrium binding isotherms were obtained monitoring sequentially the tyrosine fluorescence emission and the cysteine-Cu(I) luminescence upon addition of UAS_cL aliquots. Tyrosine fluorescence emission was quenched by 29% at saturation (Fig. 4), with its wavelength of maximum emission unchanged. Binding affinity is obviously high, resulting in a stoichiometric binding curve with an UAS_cL to ACE1 molar ratio of 0.86 (± 0.2) at the breakpoint, indicating that nearly all of the ACE1 peptide is active for DNA binding. Calf thymus double-stranded DNA did not induce tyrosine quenching of Cu-ACE1(122*) under these conditions, suggesting that the presence of the specific target sequence was necessary to induce such an effect. The UAS_cL oligonucleotide did not induce Tyr fluorescence quenching of the ACE1(122*) in the presence of 50 mM KCN, indicating that the integrity of the Cu-S cluster is necessary for specific DNA binding.

It is noteworthy that the Cu-S cluster luminescence emission was also affected upon addition of the UAS_cL oligonucleotide; a quenching of ca. 20% was observed at saturation (not shown). Again, calf thymus double-stranded DNA failed to show any reduction of Cu-S cluster luminescence intensity (not shown). This effect

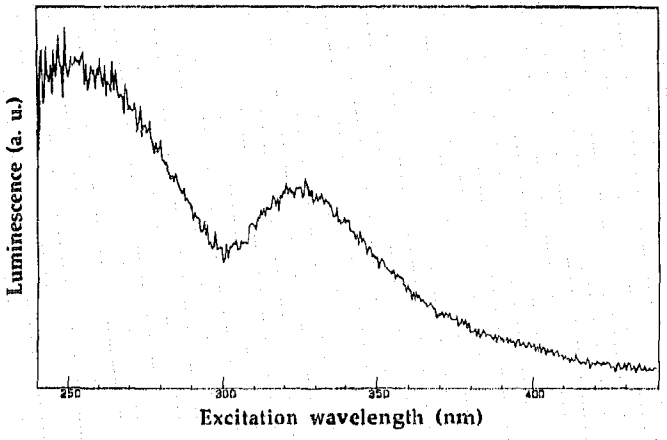

Fig. 3. Luminescence excitation spectrum (uncorrected) of ACE1(122*) (10 mM) in 5 mM potassium phosphate, pH 6.8, at 25°C. Composite of two separate scans monitoring the emission of 580 and 610 nm (8 nm bandpass), normalized at 300 nm. Excitation monochromator was set at 1 nm resolution.

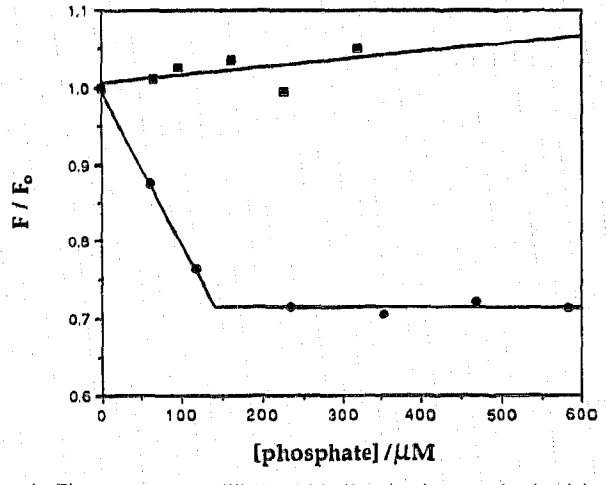

Fig. 4. Fluorescence equilibrium binding isotherms obtained in 10 mM sodium cacodylate, pH 6.15, at 25°C, monitoring the ACE1(122*) tyrosine emission intensity at 310 nm (8 nm bandpass) with excitation at 270 nm (1 nm bandpass) as a function of added aliquots of UAScL (full circles) or calf thymus double-stranded DNA (full squares).

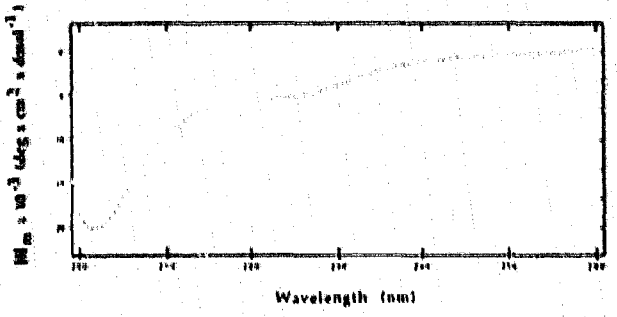

Fig. 5. Circular dichroism spectrum of ACE1(122*) (10 mM) in 5 mM potassium phosphate, pH 6.8, at 25°C. Five spectra were recorded, averaged, corrected for background and smoothed as described in the text.

was observed even with excitation at 325 nm, outside the absorption range of protein and nucleic acid chromophores, and is the first reported case of modulation of Cu(I)-thiolate luminescence by a ligand not interacting directly with the metal or sulfhydryl groups.

The CD spectrum of Cu-ACE1(122*) (Fig. 5) lacks the characteristic features of long-range α-helical or β-strand structure, probably due to the structural constraints imposed by the Cu-S clusters; it is somewhat similar to the CD spectra of compact, tightly folded proteins containing β-turns. No ellipticity was detected above 250 nm at the protein concentrations tested, suggesting a symmetric environment for the Cu(I) coordination sphere; a similar observation for Cu-metallothionein is due to its Cu(I) trigonally coordinated to sulfur.

CONCLUSION

The ACE1(122*) polypeptide, which contains the Cu- and DNA-binding domain of the yeast transcription factor ACE1, exhibits charge-transfer absorption bands and luminescence emission which are characteristic of polynuclear Cu-cysteinyl thiolate cluster. These spectral features, which are similar to those found in yeast Cu-metallothionein, disappear upon displacement of the Cu by chelators or H^+. The binding of ACE1(122*) to its specific recognition sequence UASeL, but not to double-stranded calf thymus DNA, results in quenching of the Tyr and Cu-S cluster luminescence of the polypeptide. Cu-ACE1(122*) exhibits a CD spectrum which suggests a tightly folded structure with β-bends. Our results indicate that binding of Cu(I) to cysteine residues of ACE1(122*) induces a conformation alteration required for DNA binding to its specific activator sequence.

Acknowledgements: We wish to thank Drs. E. Bucci and C. Fronticelli for access to the CD spectrometer and for their helpful assistance. Work at UMBC was supported by a grant from the American Cancer Society, No. NP-671 (to R.L.K.).

REFERENCES

[1] Thiele, D.J. (1988) Mol. Cell. Biol. 8, 2745-2752.
[2] Fürst, P., Hu, S., Hackett, R. and Hamer, D. (1988) Cell 55, 705-717.
[3] Culotta, V.C., Hsu, T., Hu, S., Fürst, P. and Hamer, D. (1989) Proc. Natl. Acad. Sci. USA 86, 8377-8381.
[4] Vandeyar, M.A., Weiner, M.P., Hutton, C.J. and Batt, C.R. (1988) Gene 65, 129-133.
[5] Studier, W.F, Rosenberg, A.H., Dunn, J.J. and Dubendorff, J.W. (1990) Meth. Enzymol. (in press).
[6] Hu, S., Fürst, P. and Hamer, D. (1990) New Biol. 2, 544-555.
[7] Van Houten, J. and Watts, R.J. (1975) J. Am. Chem. Soc. 97, 3843-3844.
[8] Demas, J.N. (1982) in: Measurements of Photoluminescence (Mislenz, K. ed.) Academic Press, Orlando, FL.
[9] Byrd, J., Berger, R.M., McMillin, D.R., Wright, C.F., Hamer, D. and Winge, D.R. (1988) J. Biol. Chem. 263, 6688-6694.