Eur Biophys J (1998) 27: 27–31

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Near-ultraviolet circular dichroic activity of apomyoglobin: resolution of the individual tryptophanyl contributions by site-directed mutagenesis

Received: 17 February 1997 / Accepted: 14 August 1997

Abstract The individual tryptophanyl contributions to the near-ultraviolet circular dichroic activity of apomyoglobin in its native conformation have been resolved by studying recombinant proteins with single tryptophanyl substitutions. Site-directed mutagenesis of sperm whale apomyoglobin was performed in order to obtain proteins containing only Trp A-5 or Trp A-12. These amino acid substitutions have very little effect on the overall globin fold as indicated by comparing the spectroscopic properties of the mutants with those of the wild type protein. The circular dichroism spectra of the two apomyoglobin mutants in the near ultraviolet were found to be significantly different, both indole residues having significant activity but of opposite sign. In particular, Trp A-5 shows the presence of a main positive peak centered near 294–295 nm with a marked shoulder at 285 nm, ascribed to the ^1L_Btransition. The spectrum of the mutant protein containing only Trp A-12 shows a large negative contribution with a minimum near 283 nm and a marked shoulder at 293 nm. The broadness of the negative contribution exhibited by Trp A-12 suggests that it may originate mainly from the ^1L_A transition.

Key words Near ultraviolet circular dichroism · Site-directed mutagenesis · Apomyoglobin · Recombinant apomyoglobin · Tryptophanyl residues

Introduction

The near ultraviolet CD activity, which many proteins exhibit between 260 and 300 nm, is mainly due to the interactions of the aromatic side chains of tryptophan, tyrosine, and phenylalanine with an asymmetric environment (Strickland 1974; Strickland et al. 1969). These interactions influence the sign and intensity of the CD bands. Moreover, the rotational strength is strongly dependent on side chain orientation and rigidity, i. e., increased mobility causes a decrease of CD activity. In this respect, the near UV CD activity is taken as an indicator of tertiary organization and, sometimes, can be helpful in detecting subtle conformational changes.

Most of the myoglobins examined so far contain two tryptophanyl residues which fill the invariant positions 7 and 14, localized in the A-helix (A-5 and A-12, respectively). Many attempts have been made to resolve the individual spectroscopic contributions of these two residues (Postnikova et al. 1991; Herskovits and Solli 1975; Kirby and Steiner 1970; Irace et al. 1981; Wasylewsky et al. 1988). These studies were mostly accomplished by selective chemical modification (Postnikova et al. 1991) or by comparing the results with those for a single tryptophan containing myoglobin, i. e., tuna myoglobin (Irace et al. 1981; Balestrieri et al. 1978). This protein possesses a single tryptophan residue, i. e., Trp A-12, the indole residue in position A-5 is missing. In previous papers, we reported that the two tryptophanyl residues exhibit different emission properties (Irace et al. 1981; Irace et al. 1986; Bismuto et al. 1989); moreover, we suggested that they might contribute differently to the near UV CD activity of apomyoglobin (Colonna et al. 1978). This result is quite surprising considering that the two residues are not only located in the same helical segment, i. e., the A helix (positions A-5 and A-12, respectively), but are also oriented on the same helical side. Since all myoglobins so far examined seem to possess the same basic fold (Rossman and Argos 1975; Argos and Rossman 1979), a comparison of myoglobins containing a different distribution of aromatic chromophores may provide information on different molecular sites despite their proximity in the primary sequence and in the tertiary organization.

In this paper, we examine the effect of single tryptophanyl replacements on the optical activity in the near UV. The residue substitutions were performed by site-directed mutagenesis of sperm whale myoglobin in order to obtain proteins containing only Trp A-5 or Trp A-12. The results

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show that the contributions of the two indole residues to the total CD activity are different.

Materials and methods

Mutagenesis of the myoglobin gene

The synthetic sperm-whale myoglobin gene (pMb 413) was kindly provided by Drs. B. A. Springer and S. G. Sligar (Springer and Sligar 1987). DNA manipulations were performed essentially as described in Sambrook et al. (1989). The Trp-7 and Trp-14 residues (Trp A-5 → Phe and Trp A-12 → Phe substitutions) were mutated with the following oligonucleotides: 5′-GTTCTGTCTGAA GGTGAATTCCAGCTGGTTCTG-3′ and 5′-GGTTCT GCATGTTTTCGCTAAAGTTGAAGCTG-3′, using the "Clontech Transformer site-directed mutagenesis" kit. Mutants were screened and confirmed by sequencing double-stranded DNA in the region of the mutation using the "Sequenase" kit purchased from United States Biochemical Corporation. The mutant myoglobin gene was expressed in a pUC19 vector in *E. coli* TB-1 (Springer and Sligar 1987).

Protein purification

Escherichia coli TB-1 harboring the pMb 413 plasmid was grown at 37 °C in LB in the presence of ampicillin (200 mg/liter). Protein was purified as described by Springer and Sligar (1987). Briefly, the cells were harvested in late stationary phase, lysed overnight and sonicated. Cell debris was removed by centrifugation, and the supernatant was brought to 60% saturation with ammonium sulfate. The precipitate was collected by centrifugation, and ammonium sulfate was added to the supernatant to 95% saturation. The precipitate was recentrifugated and resuspended in 20 mM Tris, pH 8.0 – 1 mM EDTA and applied to a Sephadex G-50 (Pharmacia) gel filtration column (2.5×100 cm). Appropriate fractions were collected and applied to a Whatman DEAE 52 ion exchange column (2.5×20 cm) equilibrated and resolved with 20 mM Tris-HCl pH 8.4. Under these conditions, myoglobin did not stick to the column and was rapidly eluted. Protein purity was checked by SDS-PAGE.

Apomyoglobin

The heme was removed from myoglobin by the 2-butanone extraction procedure of Teale (1959). The contamination of the apoprotein by myoglobin was assessed spectrophotometrically. In all cases, no significant absorption was observed in the Soret region.

The concentration of apomyoglobin was determined from absorbance at 280 nm using a molar extinction coefficient calculated from the tryptophan and tyrosine content according to Wetlaufer (1962). Concentrations were also checked by absorption methods applied in the peptide absorbing region (Scopes, 1974).

Fluorescence and polarization measurements

Fluorescence and polarization measurements were performed on a Perkin Elmer MPF-66 spectrofluorometer. Fluorescence measurements were made in the range where emission was linear with protein concentration. The absorbance of protein solutions was 0.1 at the excitation wavelength (295 nm) in a 1 cm cell. The temperature of the cell was maintained at 20 °C. The polarization was calculated from $P = (I_{VV} - GI_{VH})/(I_{VV} + GI_{VH})$ where $G = I_{HV}/I_{HH}$, I is the intensity, and the first and the second subscripts refer to the plane of polarization of the excitation and emission beams, i. e., V, vertical, and H, horizontal.

Circular dichroism

Circular dichroism measurements were performed on homogeneous samples of apomyoglobin. The absorbances of the apomyoglobin solutions were between 0.4 and 0.7 at 280 nm in 1 cm cell. A spectropolarimeter model J-710 (Jasco, Tokyo, Japan) equipped with the temperature-controlled liquid system Neslab RTE-110 (Neslab Instruments, Portsmouth, NH, USA) and calibrated with a standard solution of (+)-10-camphorsulphonic acid was used. Cuvettes (Helma, Jamaica, New York, USA) of 0.1 and 1.0 cm path length were used depending on the explored spectral range, i. e., far and near ultraviolet, respectively. A spectral acquisition spacing of 0.1 nm (1.0 nm bandwidth) was used. Each spectrum was averaged five times and smoothed with Spectropolarimeter System Software Ver. 1.0 (Jasco). All measurements were performed at the indicated temperature under nitrogen flow. The results are expressed as molar ellipticity in units of degrees $cm^2 \cdot dmol^{-1}$.

Results and discussion

We have analyzed the near ultraviolet CD activity using recombinant apomyoglobins in which one of the two tryptophan residues has been substituted with a phenylalanine residue, i. e., mutants Trp A-5 → Phe and Trp A-12 → Phe. These substitutions have very little effect on the overall globin fold: in fact, the mutated holoproteins exhibit Soret absorption and far ultraviolet CD spectra superimposible on those of wild type myoglobin. The Soret absorption maxima of the *met*-form of all three examined proteins at neutral pH was at 409 nm, a value which is coincident with that commonly found for other mammalian myoglobins (Antonini and Brunori, 1971). The far ultraviolet CD spectra of wild type and mutated myoglobins at neutral pH were found to be very similar. All spectra ex-

hibit two negative minima at 222 and 208 nm, typical of polypeptide chains in an α-helical conformation. The relative amounts of α-helical secondary structure, calculated according to Yang (1990), were 83, 83, and 82% for wild type, Trp A-5 → Phe, and Trp A-12 → Phe, respectively. This indicates that the overall secondary organization is not affected by tryptophanyl substitution. Moreover, the observation that the Soret absorption is not changed upon the amino-acid replacement, suggests that the tertiary organization of the heme surrounding is also retained.

The removal of the heme caused a decrease of negative ellipticity but did not change the shape of the far ultraviolet CD spectra of wild type and mutated apomyoglobins, which still exhibit the two typical minima centered at 222 and 208 nm. The estimated amounts of α-helical secondary structure in the three apoproteins at 20 °C were 69, 56, and 55% for wild type, Trp A-5 → Phe, and Trp A-12 → Phe, respectively. Despite the 15–20% loss of helical content, the overall globin fold is thought to be retained (Crumpton & Polson, 1965; Harrison & Blout, 1965). In fact, addition of hemin was able to produce native-like holoproteins; moreover, the three apomyoglobins were able to bind a hydrophobic probe, i. e., ANS (1,8-anilinonaphthalenesulfonate), which could be displaced by heme addition, thus indicating that the helical segments are folded to form a structurally organized heme site. The observation that the decrease in helical content is not the same for wild type and mutated proteins is not surprising: in fact, it has been reported that tuna myoglobin, a single tryptophan containing protein, exhibits a loss of helical content upon heme removal much greater than that observed for mammalian myoglobins which contain two tryptophanyl residues (Bismuto et al., 1985). However, it is possible that the amino acid substitutions may seriously affect protein stability. To check this point, we examined the thermal stability of the mutant apoproteins in comparison with that of wild type. The results indicated that the native structure of both wild type and mutants is maximally stable in the temperature range between 10° and 35 °C and denaturation occurs upon both cooling and heating.

The fluorescence emission properties of the three proteins examined further confirm that the tryptophanyl substitution, i. e., Trp A-5 → Phe and Trp A-12 → Phe, does not affect the physical properties of the surroundings of the other residue. The emission spectrum of the Trp A-5 → Phe apomyoglobin mutant is similar to that recorded for wild type apomyoglobin. In fact, both spectra showed the same emission maximum, i. e., 330.6 nm, and a similar relative intensity. The emission of the Trp A-12 → Phe apomyoglobin mutant is slightly red-shifted, the maximum being centered at 333.4, and its relative intensity is about one fourth that observed for the wild type and for Trp A-5 → Phe. The close similarity between the emission properties of wild type apomyoglobin and those of the Trp A-5 → Phe mutant further confirms that the emission of Trp A-12 is predominant in the wild type (Postnikova et al. 1991; Irace et al. 1981; Colonna et al. 1978). No significant variation of fluorescence polarization at 330 nm was observed for the three examined proteins, i. e., the values were 0.146, 0.146 and 0.157, for wild type, Trp A-5 → Phe, and Trp A-12 → Phe, respectively.

Figure 1 shows the near UV CD spectrum of mutant apomyoglobins Trp A-5 → Phe and Trp A-12 → Phe. Both spectra were recorded at neutral pH. We focused our attention on the spectral region between 270 and 300 nm, where the contributions to the CD spectrum arise mainly from tyrosine and tryptophan. Phenylalanine bands are not observed at wavelengths longer than 268 nm. The spectrum of Trp A-12 → Phe shows only positive contributions: the main peak is centered near 294–295 nm with a marked shoulder at 285 nm. The assignment of this peak could be made on the basis of the absorption and CD spectra of simple aromatic model compounds (Strickland 1974; Strickland et al. 1969; Edelhoch and Lippoldt 1968). The 294 nm peak was ascribed to one of the two vibronic components of the ^1L_B transition of tryptophan, i. e., the 0-0 transition. The second component of the couplet, corresponding to the 0 + 850 cm^{-1} transition, should be observed at 285 nm. Since the two vibronic components of the indole transition may have either positive or negative sign but not mixed (Strickland et al. 1969; Edelhoch and Lippoldt 1968), it is reasonable to attribute the shoulder at 285 nm to the second component of the couplet (Strickland 1974; Strickland et al. 1969). Although the ^1L_A transition of the indole moiety may in some cases contribute to the overall CD activity, studies performed on model compounds revealed that its contribution is much broader and generally occurs on the short-wavelength side of ^1L_B band, although some bands may appear on the long-wavelength side, especially at room temperature (Strickland 1974). Since the ^1L_A lacks any vibronic structure under most experimental conditions (Strickland 1974), it is unlikely that the positive contribu-

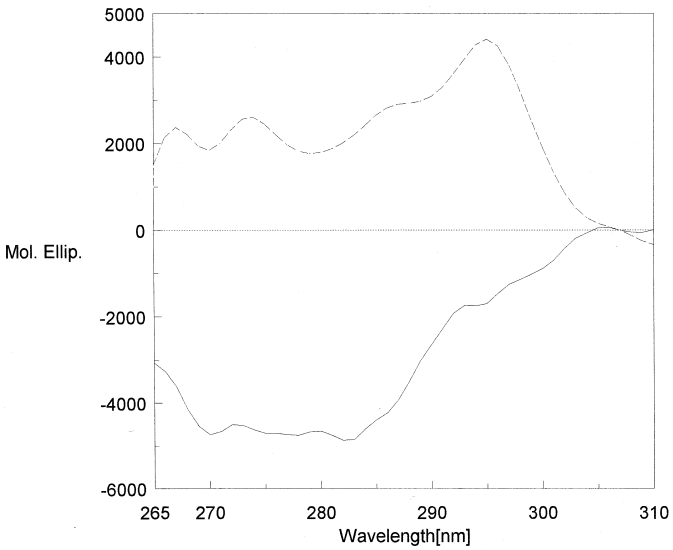

Fig. 1 Near-ultraviolet CD spectra of Trp A-12 → Phe (upper) and Trp A-5 → Phe (lower) mutant sperm whale apomyoglobins. Protein concentration was 5.0×10^{-5} M. Solvent: 0.05 M phosphate, pH 7.0. Temperature was at 20 °C. Molar ellipticity is expressed in units of deg · cm^2 · d mol^{-1}

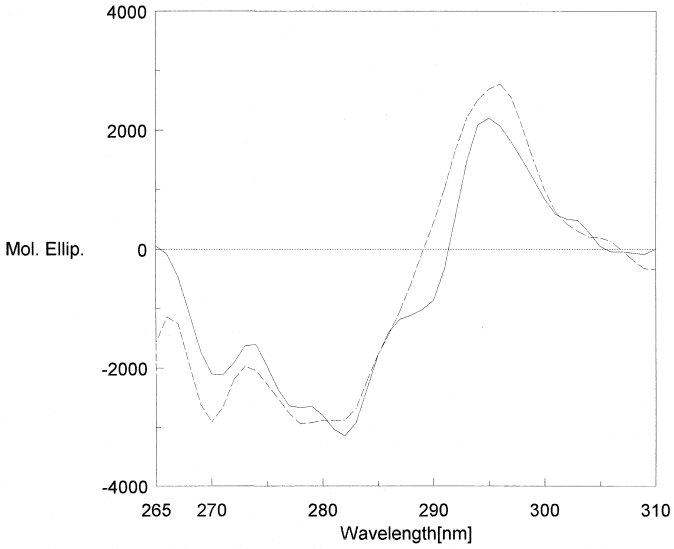

Fig. 2 Comparison between the near-ultraviolet CD spectrum of wild type apomyoglobin (–) and the computed CD spectrum obtained by summing the spectra of the two mutant apomyoglobins Trp A-12 → Phe and Trp A-5 → Phe (––). Protein concentration was 4.4×10^{-5} M; solvent: 0.05 M phosphate, pH 7.0; temperature was at 20 °C. The computed spectrum was obtained from the spectra reported in Fig. 1. Molar ellipticity is expressed in units of $deg \cdot cm^2 \cdot d mol^{-1}$

tions at 267 and 273 nm, observed in the near CD of Trp A-12 → Phe, arise from this transition.

The near UV CD spectrum of the Trp A-5 → Phe apomyoglobin mutant shows a broad negative band with minima at 283 and 276 nm and a shoulder near 293 nm. The lack of any positive contribution between 290 and 300 nm suggests that the activity of Trp A-12 differs significantly from that of Trp A-5. The assignment of the contribution arising from this residue is strongly facilitated by examining Fig. 2, where the spectrum of wild type apomyoglobin is displayed in comparison with that obtained by summing the spectra of the two mutant proteins. The close similarity between the recorded spectrum and the computed spectrum suggests that the dichroic activity of both wild type and mutants is almost exclusively due tryptophanyl residues and that these contributions are additive. Thus, on this interpretation, it is reasonable to conclude that both indole residues have significant near ultraviolet activity but of opposite sign. Both the recorded spectrum and the computed spectrum show a positive peak centered at 294–295 nm, which can be assigned to the ^1L_B transition of Trp A-5, and a large negative contribution arising from the other residue with a minimum near 283 nm and a marked shoulder at 293 nm. The broadness of the negative contribution exhibited by Trp A-12 suggests that it may originate mainly from the ^1L_A transition with a weak ^1L_B contribution at 293, 280 and, perhaps, at 270 nm.

In conclusion, the data reported in this paper clearly indicate that the two tryptophanyl residues of mammalian apomyoglobin do not possess similar spectroscopic properties despite their proximity. Both residues are in a rather hydrophobic environment as evidenced by the position of their emission maxima although with small local differences which account for the observed difference. Moreover, the two residues contribute differently to the CD activity in the near UV. The comparison of the CD spectra shown in Fig. 2 also supports the idea that the environment of these residues is not affected by the substitution Trp → Phe.

Acknowledgements This work has been supported by CNR grants # 96.00065 CT14 and 95.02698CT14.

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