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DISTAL HIS→ARG MUTATION IN BOVINE MYOGLOBIN RESULTS IN A LIGAND BINDING SITE SIMILAR TO THE ABNORMAL BETA SITE OF HEMOGLOBIN ZURICH (β63 HIS→ARG)

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Summary Carbon monoxide binding to a myoglobin mutant with distal arginine in place of histidine has been examined. The mutant is derived from a cDNA clone for Mb mRNA from fetal bovine skeletal muscle. The mutation only slightly perturbs visible/Soret spectra whereas the infrared spectrum of liganded CO is greatly modified to become nearly identical to Hb Zurich β-subunit spectrum. The mutant IR spectra differ substantially from spectra of wild-type MbCO and normal HbCO β-subunit. For both the Mb and the Hb the distal His→Arg mutation increases the affinity for CO and reduces the number of observed conformers. These results demonstrate that this mutation greatly reduces the differences between Mb and Hb in the structure and properties of its ligand binding sites.

Introduction In accordance with their respective physiological roles, myoglobins typically differ substantially from hemoglobins in affinities of O_2, CO and other ligands and in the ligand binding site structures found in crystal structures (1,2). Infrared spectra of bound CO reflect these differences in structure and demonstrate the dynamics of ligand site conformers, two major conformers for myoglobins and one major conformer per normal hemoglobin subunit (3-6). The availability of Hb Zurich, a mutant human adult hemoglobins with arginine in place of distal (E7) histidine, permitted an evaluation of the influence of the distal histidine on normal structure and function of HbA. The mutation results in lowered thermal stability, greater affinity for CO, and greater reactivity of bound O_2 toward reductants due to structural changes apparent in a crystal structure (7) and in infrared spectra for O_2 and CO ligands (6-8).

The unavailability of suitable mutants has precluded similar studies of the myoglobin ligand site. Recently a method was developed for the expression of bovine myoglobin cDNA as a functionally active holoprotein in Saccharomyces cerevisiae making possible the replacement of distal histidine with arginine by site-directed mutagenesis (9). We report here effects of this myoglobin mutation on CO infrared spectra and CO affinity that are similar to those

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observed earlier for Hb Zurich. With arginine in place of distal histidine the differences in ligand site structure between the myoglobin and the hemoglobin are greatly reduced.

Materials and Methods *Preparation of myoglobin solutions.* Wild-type bovine heart MbO_2 was obtained as described previously (4). The E7Arg bovine MbO_2 mutant was prepared via site-directed mutagenesis methods described elsewhere (9). The carbonyl complexes were obtained from exposure of an MbO_2 solution in 0.1M Tris buffer pH 8.5 to CO; a small amount of sodium dithionite was added to reduce any metMb present.

Measurement of spectra. The CO-IR spectra of MbCO solutions in a Beckman FH-01 cell with CaF_2 windows and 0.1 mm pathlength were determined with a Perkin-Elmer Model 1800 Fourier transform infrared spectrophometer with an Hg/Cd/Te detector. A 1000 scan interferogram was collected in single beam mode with 2 cm^{-1} resolution and a 1 cm^{-1} interval. MbO_2 spectra obtained under identical conditions were used as reference spectra. Visible/Soret spectra were obtained with a Varian Model 2200 spectrophotometer.

Results *E7His→Arg effect on visible/Soret spectra.* The mutant MbCO exhibits α and β visible bands at 576 and 540nm, respectively, compared with 577 and 539nm for the wild-type MbCO. The Soret maximum is red shifted 1nm in the mutant spectrum (Fig. 1).

E7His→Arg effect on C-O stretch bands. The mutation caused a 13.5 cm^{-1} blue shift in absorbance maximum and a change in the shape of the absorption envelope (Fig. 2). The major band of the E7His MbCO spectrum is asymmetric toward the red; earlier deconvolutions indicate a narrow ($\Delta\nu_{1/2}$=9 cm^{-1}) band (CII) centered at 1943.5 cm^{-1} and a wide ($\Delta\nu_{1/2}$=18 cm^{-1}) band (CI) at 1938 cm^{-1} (4). In contrast the E7Arg MbCO spectrum consists almost entirely of a single narrow ($\Delta\nu_{1/2}$=8 cm^{-1}) band centered at 1957 cm^{-1}; a shoulder indicates a contribution from a weaker band near 1965 cm^{-1}.

E7His→Arg effect on CO affinity. A solution equimolar in mutant and wild-type Mbs partially saturated with CO gave the spectrum A of Fig. 2. The greater intensity at 1957 cm^{-1} than at 1943.5 cm^{-1} shows the greater affinity of the mutant for CO; about 7 μM MbCO (E7His) and 20 μM MbCO (E7Arg) are present. Spectrum B represents the difference spectrum from subtraction of normalized 1957 cm^{-1} band of MbCO(E7Arg) from spectrum A.

Discussion *CO-stretch bands and ligand site conformers.* The infrared spectrum of bovine heart MbCO in the C-O stretch region can be deconvoluted into four bands (Table 1)(3,4). Each band has been attributed to a discrete conformer structure at the ligand binding site. Two bands (CI and CII) of

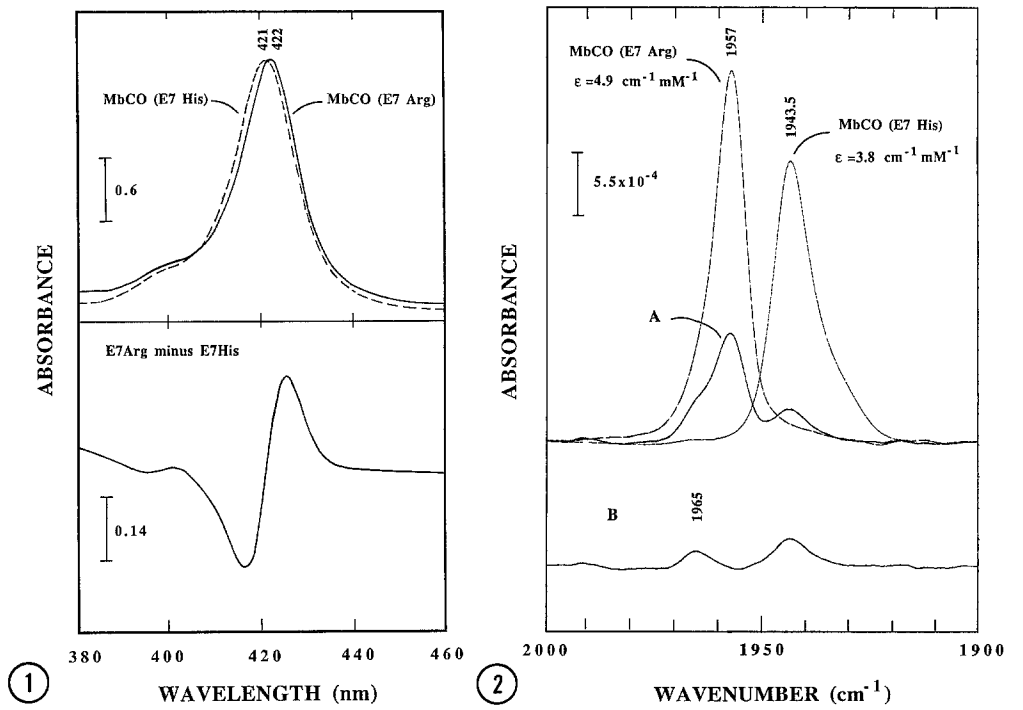

Figure 1. Soret spectra of bovine heart Mb (wild-type) and mutant bovine Mb (E7His→Arg) carbonyls. (Upper panel): Mutant MbCO ——, wild-type MbCO ---. (Lower panel): Difference spectrum: mutant MbCO spectrum minus wild-type MbCO spectrum of upper panel. All spectra were measured in solutions of 0.1M Tris buffer, pH 8.4 at 20°C.

Figure 2. Infrared spectra of carbon monoxide liganded to myoglobins. (——) Mutant bovine Mb(E7His→Arg), 67 μM. (----) Wild-type bovine MbCO, 67 μM. (A) Solution containing 67 μM mutant Mb, 67 μM wild-type Mb, and 27 μM CO. (B) Difference spectrum obtained by digital subtraction of the major band at 1957 cm^{-1} for the mutant MbCO from spectrum A. All spectra were measured in solutions of 0.1M Tris buffer, pH 8.4 at 20°C.

Table 1
Parameters of Deconvoluted C-O Stretch Bands for Infrared Spectra of Bovine Myoglobin and Human Hemoglobin

	Conformers							
	CI		CII		CIII		CIV	
Protein Carbonyl	$\nu_{CO}(\Delta\nu_{1/2})$ cm^{-1}	area %	$\nu_{CO}(\Delta\nu_{1/2})$ cm^{-1}	area %	$\nu_{CO}(\Delta\nu_{1/2})$ cm^{-1}	area %	$\nu_{CO}(\Delta\nu_{1/2})$ cm^{-1}	area %
MbCO[a] (wild)	1938(18)	48	1944(9)	46	1953(9)	3	1965(10)	3
MbCO (E7His→Arg)	b		b		1957(8)	91	1965(8)	9
Hb Zurich[c] (β E7His→Arg)	1934(8)	1	1943(7)	4	α1950(7) β1958(8)	50 39	1970(7)	4
Hb A[c]								
α-subunit	1934(8)	1	1944(8)	5	1951(7)	91	1969(9)	1
β-subunit	b		b		1952(7)	99	1969(9)	1

[a] See reference 4. [b] Not observed. [c] Reference 6. [c] References 6 and 13.

nearly equal integrated intensity comprise >90% of the total absorbance. Relative band intensity indicates relative conformer stability. Changes in temperature and pH near 20°C and pH 7 alter relative band intensities (and relative conformer stabilities) with little change in band frequency or width and no change in total integrated band intensity (3.4).

Four bands with parameters similar to those for bovine MbCO have been found for carbonyls of myoglobins with diverse amino acid sequences from 13 species (10). However, the E7His→Arg mutation in bovine Mb results in a very different spectrum dominated by a band at 1957 cm^{-1} and a shoulder to the blue due to a weaker band at 1965 cm^{-1}; thus only one conformer of high stability and one of low stability are indicated. The low stability (1965 cm^{-1}) conformer is more stable in the mutant than the E7His MbCO. The structures and dynamics of the ligand site are therefore changed markedly by the E7His→Arg substitution.

The resulting CO-infrared spectrum is very similar to the spectrum of the Hb Zurich CO β-subunit (Table 1)(6). However, the blue shift in the spectrum for the Hb β-subunit (1951→1958 cm^{-1}) that accompany E7His→Arg are smaller than for the Mb (1938, 1944→1957 cm^{-1}).

E7His→Arg effects on CO affinity. Spectrum A of Fig. 2 demonstrates a nearly three-fold greater binding of CO to the E7Arg mutant than to E7His Mb. The abnormal β-subunit of Hb Zurich also binds CO more avidly than the normal β-subunit of HbA. This property significantly affects the clinical manifestations of Hb Zurich disease, especially for patients that are cigarette smokers (11).

E7His→Arg effects on ligand site structures. The spectra of Fig. 2 reflect changes in ligand site structure of bovine MbCO due to replacement of distal histidine by arginine. The number of observable conformers is reduced from 4 to 2. For reasons discussed earlier (3-5) the mutant major band at 1957 cm^{-1} is expected to represent a CO ligand more nearly normal to the porphyrin plane than in CI, CII, or CIII of the E7His MbCO; with distal Arg present little steric constraint, if any, appears to be placed on CO to cause bending and/or tilting of Fe-C-O bonds. The relatively narrow band widths of the mutant MbCO bands (8 cm^{-1} for both 1957 and 1965 cm^{-1} bands) are comparable to those for CII, CIII and CIV bands, and narrower than the CI band, of E7His MbCO (Table 1). Clearly the relief from steric constraints has not induced greater band broadening due to a loosening of the ligand environment, nor has the ligand become exposed to external medium (12). Increased mobility (flexibility) of the groups comprising the immediate environment of CO is expected to increase band widths. The dynamics of ligand-environment interactions appear comparable in E7Arg MbCO to those in HbACO, Hb Zurich CO, and CII, CIII, and

CIV, but not CI, of E7His MbCO (Table 1). These data suggest the crystal structure for the ligand site of the β-subunit of Hb Zurich CO (7) also represents a useful model for the site in E7Arg MbCO. Comparisons of CO-IR spectra support a β-subunit ligand site in solution similar to the crystal structure of Hb Zurich CO (6). However, in some cases, as shown in CO-IR spectra for the β-subunit of HbACO, there can be a distinct difference in crystal and solution structures (6).

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