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ABSORPTION SPECTROSCOPY AND CIRCULAR DICHROISM STUDIES OF SPERM WHALE MYOGLOBIN CHEMICALLY MODIFIED ON THE N-TERMINAL α-AMINOGROUP BY ISOTHIOCYANATE REAGENTS

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Sperm whale metMb [Mb(SW)] was modified chemically by fluorescein isothiocyanate and methylisothiocyanate. Individual modification products on the α-aminogroup of the N-terminal Val were isolated with ion exchange chromatography (FITC-Mb and MITC-Mb). Absorption spectra in the 200–700 nm region and spectrophotometric titration curves in the Soret band of the modified metMb derivatives and intact metMb were compared. Characteristic differences between them indicate that upon modification there occurs a shift in the equilibrium of isomers of the metMb aquo complex towards the low-spin form. The CD spectra of FITC-metMb and MITC-metMb in the 200–450 nm region attest to small changes in the heme environment as compared to native metMb without, however, any appreciable conformational changes of the polypeptide chain. No differences have been found in the absorption and CD spectra in the Soret region between native deoxy-Mb and the modified Mb derivatives in deoxy forms. An analysis of the present results and of those reported in the literature shows that the conformational changes at the N-end of Mb upon modification of the N-terminal α-amino group result in structural alterations in the heme environment which are most likely to consist in some reorientation of the side group of His E7 and, possibly, those of Phe B14, Phe CD1, and Phe CD4 on the distal side of the heme. A scheme of the electronic conformational interactions (ECI) in ferrimyoglobin is proposed.

1. Introduction

It is evident from the three-dimensional structure of sperm whale myoglobin that electrostatic interactions play an important role in maintaining the native conformation of the N-terminal region of the molecule [1]. The two "salt bridges" formed by the COO^--groups of Glu A2 and Glu A4 with, respectively, the NH_3^+-groups of Lys EF2 and Lys H10 serve to stabilize the N-end of the A-helix.

The electron density of the amino terminus was too weak to fix the orientation of Val NA1. But from a spin-labeled Mb study it was shown that the N-terminal α-NH_3^+-group also participates in ionic interactions at the N-end to preserve the structure of this part of the molecule [2]. The spin-label attached to His A10 registrated pH-induced conformational transition in met-Mb (with pK 7.8) and in CN-Mb (with pK 7.4) which was due to deprotonation of the Val NA1 α-amino group. This transition was absent if the α-NH_2 group was specifically modified by methylisothiocyanate [3]. Localisation of the spin-label at His A10 supported the idea that upon the α-NH_2 group deprotonation a relative position of the A-helix and GH-fragment has being changed.

Another interesting result as could be seen from the different pK values for the transition in metMb and CN-Mb was a conformational change of the N-terminus when the H_2O molecule in the sixth ligand position of the heme was replaced with CN-ion. Unlike this the spin-labels bound to the tyrosine residues HC3 and G4 did not register any pH- and ligand-induced conformational transitions in the C-end part of myoglobin [2,3].

In the present paper an influence of changing the

ionic interactions and the conformation at the N-terminus on heme properties in myoglobin was investigated. For this purpose the Val NA1 α-amino group was modified chemically by fluoresceinisothiocyanate (FITC) and methylisothiocyanate (MITC). Isothiocyanates have been shown to react selectively with the α-NH_2 groups of proteins under controlled pH [4—7]. Individual modification products (FITC-Mb and MITC-Mb) were isolated using ion exchange chromatography and thiocarbamylated amino acid residue was identified in them.

Then absorption and CD spectra of the modified myoglobins in met and deoxy forms were compared to those of the native Mb, and also their curves of spectrofotometric titration in the Soret band. The results obtained show that the chemical modification results in some alteration of heme characteristics in metMb which are proved to connect with a changed orientation of the protein groups from the distal side of the heme.

2. Materials and methods

2.1. Materials

Myoglobin was isolated from sperm whale skeletal muscle by extraction and fractionation with ammonium sulfate followed by purification with ion exchange chromatography on CM-Sephadex C-25 [8].

Fluoresceinisothiocyanate (Serva, USA) was used without further purification. Methylisothiocyanate was a gift from Professor V.M. Stepanov and was synthesized in the Laboratory of Protein Chemistry, Department of Chemistry, Moscow University. Its boiling point was 232°C.

2.2. Chemical modification

The reaction of metMb with FITC was carried out in the presence of a 5-fold molar excess of the reagent in 0.1 M phosphate buffer, pH 7.0, at +5°C. After 72 h the excess of reagent was removed by gel filtration through Sephadex G-25 after adding a small amount of $K_3[Fe(CN)_6]$. The reaction mixture was then fractionated on a CM-Sephadex C-25 column with a stepwise gradient from 0.01 to 0.1 M phosphate buffer, pH 6.5. In a small fraction eluted by 0.01 M buffer two dye molecules were bound to a protein molecule. The main fraction of modified Mb eluted with 0.05 M buffer concentration was monomodified product (FITC-Mb). A yield was about 10% of metMb taken into the reaction. The number of molecules of the dye in FITC-Mb was determined spectrophotometrically at pH ⩾ 8 using $\epsilon_{495} = 72\,000\,M^{-1}\,cm^{-1}$ [7].

The chemical modification of metMb by MITC was carried out with a 10-fold molar excess of the reagent in 0.1 phosphate buffer, pH 6.5, at +5°C for 72 h. After an intensive dialysis with the addition of $K_3[Fe(CN)_6]$, the reaction mixture was chromatographed on a CM-Sephadex C-25 column. The elution was carried out with a stepwise gradient from 0.01 to 0.1 M phosphate buffer, pH 6.4. The main fraction of the modified protein eluted with 0.05 M buffer (MITC Mb) was collected. An yield of MITC-Mb was about 40% of the initial Mb amount.

2.3. Analyses of the N-terminal sequence

Heme group was extracted from FITC-Mb and MITC-Mb according to a standart method [10]. Modified apoproteins were then lyophilized.

The N-terminal amino acid sequence was determine by automated Edman's phenylisothiocyanate method on a Beckman 890 C sequenser (USA). Program was started with a cleavage of the N-end residue and then nine full cycles more were run to identify the N-terminal sequence of Mb. PTH-derivatives of amino acids were identified by thin-layer chormatography on silica gel and by gas-liquid chromatography on a Hewlett-Packerd 5710A gas chromatograph. For evaluation of α-NH_2-group modification extent relative amounts of Val NA1 and Leu NA2 PTH-derivatives after the secon cycle were compared. In MITC-apo Mb less than 10% of Val PTH-derivative was found which corresponded to 95±5% modification of N-terminal amino group. According to amount to about 25% of Val PTH-derivative found in FITC-apo Mb after second cycle the modification excent was rather in 80 ± 5% range. The point is that fluorescein TH-derivative can not be so readily recoved in ethyl acetate extract as methyl and phenyl TH-derivatives. Besides, acid conditions of degradation were shown to lead to the partial recovery of free amino acids from their fluorescein thiocarbamy and thiohydantoin derivatives [5,7,11].

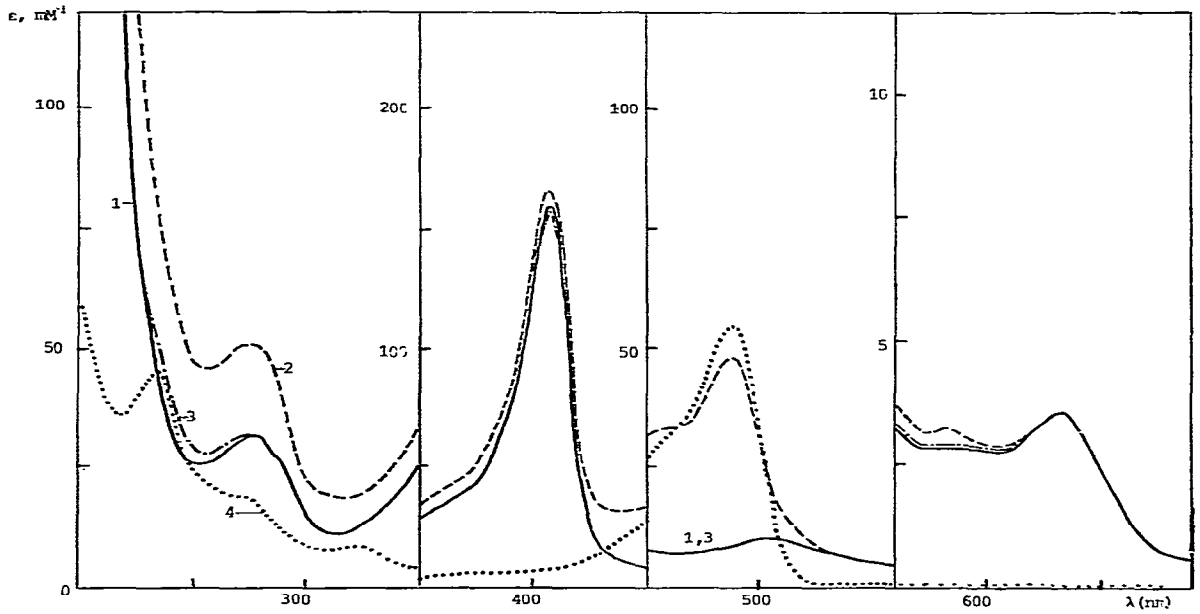

Fig. 1. Absorption spectra in the ultraviolet, Soret and visible regions: 1, native metMb (——); 2, FITC-metMb (— — —); 3, MITC-metMb (— • —) and 4, fluorescein (• • •). 0.01 M phosphate buffer, pH 6.5.

2.4. Optical measurements

The absorption spectra in the UV and visible regions were recorded with a Specord UV VIS spectrophotometer (GDR). The concentration of metMb was determined using $\epsilon_{409} = 160\,000\,M^{-1}\,cm^{-1}$.

The reduction of ferric Mb derivatives at different pH values was accomplished with sodium dithionite under the lowered pressure of 2.5×10^{-2} mm Hg to avoid contact with oxygen of the air.

Spectrophotometric titration was performed in the Soret band in the pH range 6 to 11 at 22 ± 0.5°C. The Mb concentration in 0.01 M phosphate buffer was $5-7 \times 10^{-6}$ M/l. The pH was adjusted by carefully adding NaOH solution with vigorous stirring. The dilution of the titrated solution did not exceed 1% of the starting volume. pH was measured on a LPU-01 pH-meter to an accuracy of ±0.05 pH unit before and after recording the spectrum.

2.5. CD measurements

CD spectra of Mb derivatives were registered with a J-20 spectropolarimeter (Jasco, Japan) in cells of various pathways (1 to 5 mm). The protein concentration was 0.3–1.5 g/l in 0.01 M phosphate buffer, pH 6.5. Spectra were recorded at least for 2–3 different concentrations. No effects reported by Sjöholm and Ekman [9] were observed in our experiments. The instrumental error at the maximal effect was not greater than 1–1.5%. The errors indicated in the figures are greater than the instrumental ones, being 5–10% of the maximal effect. These errors were due to those associated with calibration of the instrument, inaccuracies in determining the concentration of a protein solution, and so on.

The specific ellipticity for a peptide chromophore was determined taking into account the Lorentz factor by the formula

$$[\theta']_{MRW} = \frac{3}{n_\lambda^2 + 2} \frac{10\,MRW}{cl} \varphi ,$$

where MRW is the mean molecular weight of the amino acid residue in Mb equal to 116, φ is the ellipticity in degrees, n_λ is the refractive index of the solution at the wavelength λ, c is the protein concentration in g/l, and l is the optical path in dm. In the spectral region 250–450 nm, the molar ellipticity $[\theta']_M$ was used instead of

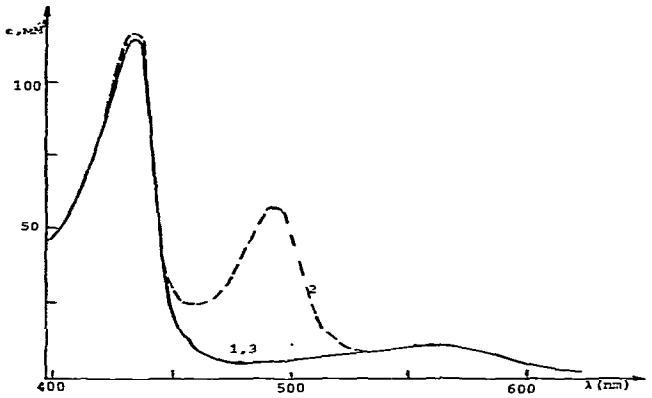

Fig. 2. Absorption spectra of deoxy derivatives of native Mb, FITC-Mb, and MITC-Mb in 0.01 M phosphate buffer, pH 7. Designations as in fig. 1.

$[\theta']_{MRW}$ as the effect in this case is not the result of averaging for all residues but pertains to the molecule as a whole.

The CD spectra of ferrous Mb derivatives were measured in hermetically sealed cells after reduction with sodium dithionite.

3. Results and discussion

3.1. Absorption spectra in the UV, Soret and visible regions

Fig. 1 presents absorption spectra of modified metmyoglobins and native metMb in the 200–700 nm region at pH 6.5. It also shows the spectrum of fluorescein at the same pH.

It can be seen that in the 200–550 nm region the FITC-Mb spectrum (curve 2) differs significantly from the Mb spectrum (curve 1) due to absorption of the dye (curve 4). It should be noted that the binding of fluorescein to Mb results in a reduction of about 20% in the absorption coefficient of the dye at 495 nm, which is in agreement with the data for other proteins [7].

The most remarkable difference between FITC-Mb and Mb spectra is seen in the 560–620 nm region where the dye does not absorb. An increase in absorption in this region with the maximum at about 580 nm is characteristic for the low-spin Mb complexes and points that in FITC-metMb an equilibrium of high- and low-spin isomers of the aquo complex is shifted toward the low-spin form. The gain in FITC-Mb absorption at 580 nm corresponds to an increase of about 10% in the low-spin form, as compared to the content of this form in hydroxy Mb at pH 10 [10].

The MITC-Mb spectrum (fig. 1, curve 3) is not noticeably different from that of native metMb in the Soret and visible regions while in the UV region there is a slight increase in absorption in the 220–280 nm interval.

It has been shown by difference spectroscopy that the gain in absorption in this region with the maximum at 240 nm is characteristic for low-spin derivatives of ferrimyoglobin [12]. The authors attributed this change to an electronic transition arising from the heme-linked imidazole ring. One may therefore conclude that in MITC-Mb, too, the equilibrium is shifted towards the low-spin complex, though to a smaller degree than in FITC-Mb. The possible changes of this type in the FITC-Mb spectrum in the UV region are masked by absorption of the dye.

Fig. 2 shows the spectra of sodium dithionite-reduced MITC-Mb, FITC-Mb, and native Mb in the Soret and visible regions. The MITC-Mb (2) spectrum is completely identical to that of intact deoxy-Mb (curves 1 and 3). The FITC-Mb (2) spectrum shows, in addition, an absorption band with the maximum at 495 nm which belongs to the dye. Like the native Mb (2) the spectra of modified deoxy-Mb derivatives are pH independent in the PH range 6.5 to 10.1.

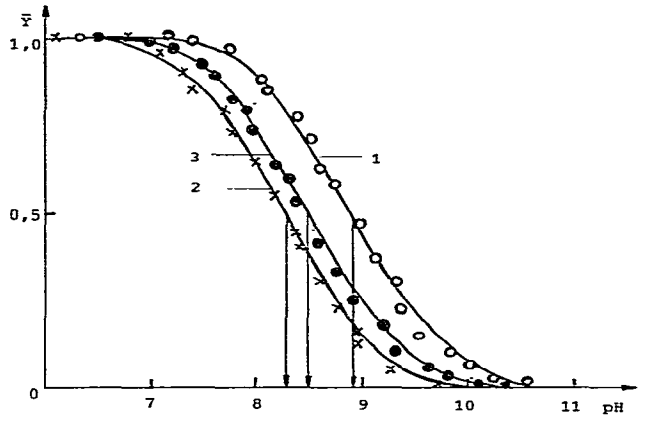

Fig. 3. Spectrophotometric titration in the Soret band of: 1, native metMb (○), 2, FITC-metMb (×), and 3, MITC-metMb (●). 0.01 phosphate buffer, 22 ± 0.5°C. $\bar{Y}$ is the relative change of optical density. The solid lines are theoretical ionization curves of the groups ($\Delta n_{H^+} = 1$) with the pK values indicated.

3.2. Spectrophotometric titration in the Soret band

Fig. 3 shows spectrophotometric titration curves at 409 nm for met derivatives of FITC-Mb, MITC—Mb and native Mb in the pH range 6 to 11.0.

While native Mb has been reported [10] to show a met-hydroxy transition with the pK = 8.9 (curve 1), the ionization pK of a liganded water molecule is 8.3 for FITC-Mb (curve 2) and 8.5 for MITC-Mb (curve 3).

The observed pK shift towards more acid pH values suggests that the chemical modification elicits a conformational change in heme environment such that it facilitates the ionization of the H_2O molecule in the sixth coordination position of the Fe atom. This change is more considerable in the case of FITC-Mb, which is consistent with the above data on absorption spectra.

3.3. CD spectra

3.3.1. Soret region (350—470 nm)

The circular dichroism of Mb in this region reflects the optical activity of the heme group. Since the heme itself is optically inactive, an induced Cotton effect arises owing to an asymmetric protein environment, mainly as a result of interaction between electronic transitions of the heme (B-transitions) and the allowed π—π^* transitions in the nearby aromatic side groups [13]. The orientation of these groups relative to the heme strongly affects the CD spectrum in the Soret region, which makes the latter a highly sensitive probe for changes in the heme environment.

Fig. 4a presents the CD spectra of native metMb (curve 1) and of FITC- and MITC-modified metMb derivatives (curves 2 and 3, respectively) in the 350—470 nm region. Also shown are the CD spectra of deoxy forms of native Mb and MITC-Mb (curves 1′ and 3′ respectively) after reduction by sodium dithionite. Ellipticity values for both met- and deoxy derivatives of native Mb agree with those reported by Nicola et al. [14].

It can be seen that the CD spectra of reduced Mb(2) and MITC-Mb(2) completely coincide (curves 1′ and 3′), which seems to indicate that the heme environment is the same in both cases.

However, for metMb derivatives the band intensity at the maximum of the effect (405 nm) is approximately 20% less with MITC-Mb and FITC-Mb than with native Mb; positions of the band maxima remain unchanged in all the samples examined. A shape of the Soret CD band in ferriMb may vary upon the binding of different ligands [14], but under experimental conditions (pH 6.5) the H_2O molecule must be retained in the sixth ligand position in both modified metmyoglobins.

The most plausible explanation for differents ellipticities of modified and native metmyoglobins in the Soret band is that orientations of chromophore groups in the heme environment undergo some alterations as a result of the modification [13]. A change in heme orientation is unlikely in view of its rigid fixation within the Mb structure [1,15].

3.3.2. Near-ultraviolet region (250—320 nm)

The CD spectra of the met forms of FITC-Mb, MITC-Mb, and native Mb in this region are presented in fig. 4b.

In the 245—280 nm interval the MITC-Mb spectrum (curve 3) differs somewhat from one of the native protein (curve 1) but coincides with it in the 280—305 nm interval.

It is very difficult to interpret the CD spectra and the changes therein in the near-ultraviolet region because the dichroic contributions of aromatic chromophores and the L- and N-transitions of the heme overlap

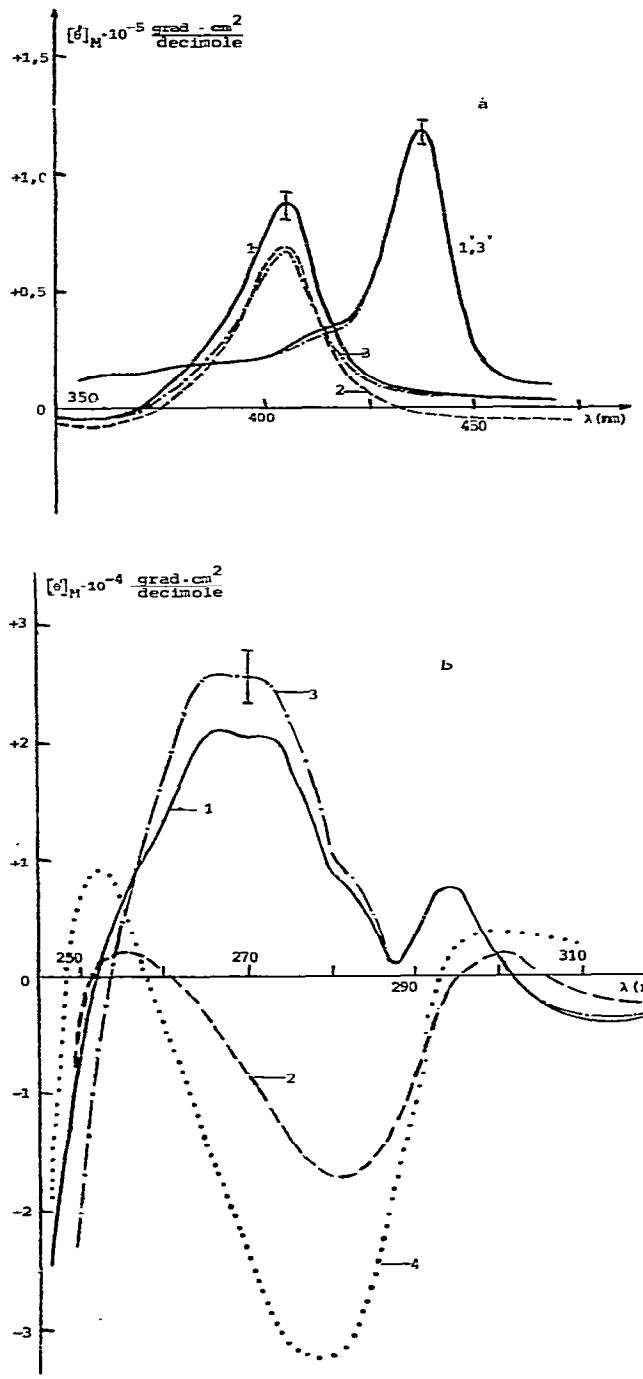

Fig. 4. CD spectra of native Mb and FITC- and MITC-modified Mb derivatives in the Soret (a), near-ultraviolet (b), and far-ultraviolet (c) regions. 0.01 phosphate buffer, pH 6.5. 1, native metMb (——); 2, FITC-metMb (– – –); 3, MITC-metMb (– · –); 4, FITC-metMb, Fraction I (·····). 1' and 3', the spectra of ferrous derivatives of native Mb and MITC-Mb respectively in the Soret region.

in this region [13,14,16]. However, it has been shown by CD studies of various heme proteins and Trp derivatives as well as on the basis of theoretical consideration that a band series near 292 nm is due to Trp chromophores that occur in different environments [14,17,18] Since the 280–305 nm region shows no differences in CD between MITC-Mb and native Mb, this means that the modification does not alter substantially either orientation or an environment of the two Trp residues of the A-helix.

A small increase in MITC-Mb ellipticity in the 250–280 nm region can be presumably attributed to a chang in the rotational strength of the heme L-band because of altered orientation of aromatic chromophores in the heme environment.

Note that the CD spectrum of FITC-Mb (curve 2, fig. 4b) differs sharply from those of Mb and MITC-Mb by displaying a large negative effect with the maximum near 280 nm. Comparison with the CD spectrum of the modified Mb with the two bound FITC-molecules

(curve 4), where the corresponding effect is twice as large, suggests that this effect is brought about by induced optical activity of the dye [19]. Free fluorescein is optically inactive.

3.3.3. Far-ultraviolet region (200–250 nm)

In this region CD spectra provide information about the conformational state of a polypeptide chain. Comparison of CD spectra of native metMb, FITC-metMb, and MITC-metMb shows that the spectra of all specimens coincide within the experimental error (fig. 4c, curves 1–3). It follows that the chemical modification does not result in any appreciable conformational changes in the polypeptide chain. The α-helix content as calculated from the specific ellipticity at 222 nm [14], is 77%, which is in agreement with X-ray data.

3.4. Electronic conformational interactions in myoglobin

The effect of chemical modification of the N-end α-amino group which is about 20 Å away from an "active centre" on the latter's environment serves to demonstrate an important regulatory role of protein structure and may have functional implications. To gain an understanding of the changes that occur near the heme group, an attempt has been made to analyze the present findings in conjunction with those from other studies of ligand- and pH-induced conformational changes in Mb using other techniques, primarily X-ray analysis.

The rotational strength in the Soret region is, as already indicated, the resultant of many interactions between the heme and surrounding amino acid residues. According to theoretical calculations, of the twelve side groups of Tyr, Phe and His situated in Mb within of 12–15 Å from the Fe atom, the heme interactions with Phe B14, Tyr G4, His E7, His FG2, Tyr H23, Phe H15, Phe G7, Tyr HC2, Phe CD4, and Phe CD1 contribute most to an induced Cotton effect in the Soret band [13]. Inasmuch as the titration data of FITC-Mb and MITC-Mb indicates that changes tend to occur in the heme cavity, is close proximity to the heme, Phe B14, His E7, His FG2, and Phe CD1 ($r < 7$ Å) may well be regarded as the residues most likely to be responsible, due to changes in their orientation, for differences in CD spectra (fig. 4a). It is to be noted that the heme interaction with Phe CD1 also makes the largest contribution to the heme L-band with the maximum near 265 nm [13].

The remaining chromophore groups are much farther away from the heme group (10 to 15 Å) so that the possible alterations in their position should affect CD in the Soret band to a lesser extent. Also, chemical modifications of Tyr G4 and Tyr HC2 are known not to alter the spectral properties or pK values of the methydroxy transition in metMb. The imidazole group of the proximal His F8, although placed very close to the heme ($r = 3.6$ Å), occupies a rather symmetrical position and contributes but slightly to the induced Cotton effect which virtually unchanged as the plane of the imidazole ring is rotated from 0° through 90° or as its inclination relative to the heme plane is varied 1° through 10° [13].

Furthermore, as shown above, changes in absorption and CD spectra occur only for the met forms of FITC-Mb and MITC-Mb when the sixth coordination position of the Fe atom is occupied by H_2O molecule and are not observed in deoxy derivatives when no sixth ligand is present and its site remains vacant. Hence it may be concluded that conformational changes in the heme cavity primarily effect the area of contacts of the sixth ligand, i.e., the destal His E7 and the adjacent portion of the E-helix. When metMb converts to deoxyMb and the liganded H_2O molecule is displaced from its position the number of heme contacts on the distal side should decrease and the differences between native Mb and modified Mb derivatives should diminish especially if conformational rearrangements in this particular region upon the modification have the same direction as upon reduction of Mb.

Recent X-ray studies by Takano [15] have shown that heme contacts with His E7 and the Phe CD1 do strongly decrease on transition from met- to deoxy-Mb. Also, there occur shifts in the first 13 residues of the E-helix. After being freed from its binding with the liganded H_2O molecule and sulfate ion the distal His E7 imidazole, which like a door blocks the entrance to the heme pocket in MetMb, shifts from its position towards the hydrophobic cluster near His E7. This cluster includes the side groups of Phe B14, Phe CD1, and Phe CD4 mentioned above. The fact that the ionization of the heme-linked H_2O molecule in FITC-Mb and MITC-Mg is facilitated suggests the possibility of His E7 making a similar shift as a result of the modification.

Finally, the modification of the α-amino group by FITC or MITC has shown to shift the equilibrium Mb

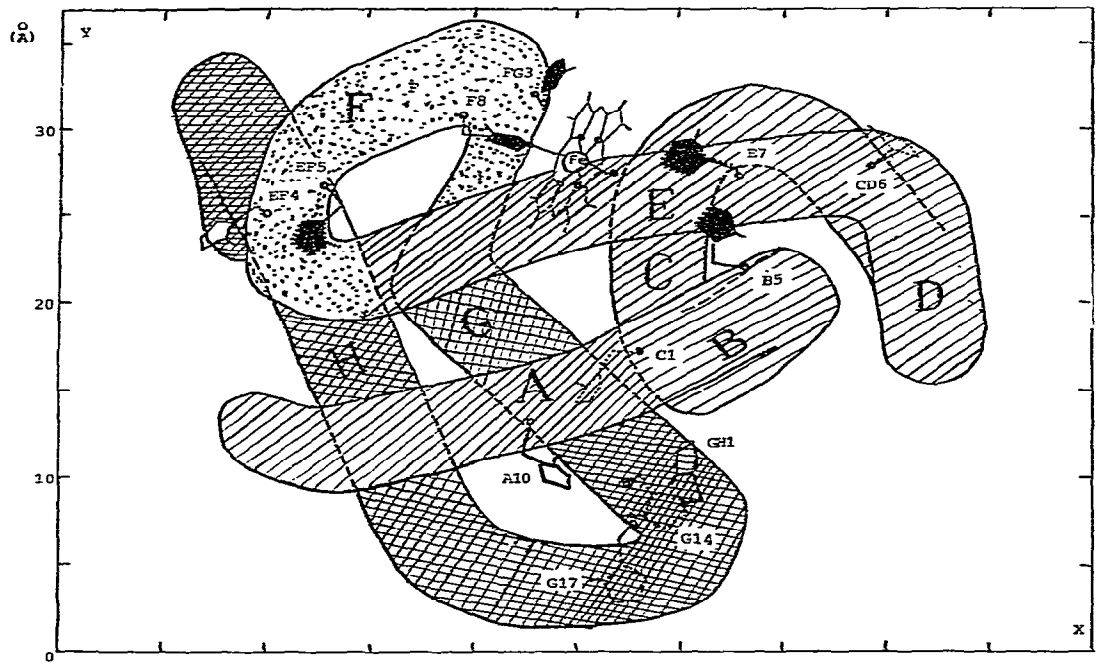

Fig. 5. Three-dimensional structure of sperm whale myoglobin in projection XY. There are indicated the positions of titratable (open) and untitratable (shaded) His residues in metMb [20].

aquo complex isomers towards the low-spin form. It follows from NMR titration data for His residues of the met and azide myoglobins [20] that formation of the low-spin complex does not reveal any conformational changes in the vicinity of His G14, His G17 and His EF4 but is accompanied by conformational changes near His C1, His CD6, His B5, and possibly His GH1 and His A10 (fig. 5). This is in agreement with the difference electron density maps of the cyanide, azide and hydroxide complexes of ferrimyoglobin versus metMb [20–22]. X-ray diffraction analysis shows, in addition, small conformational rearrangements near Lys B15 and Ser B16 as well as in the G-helix, but the largest conformatinaol changes are seen in the region of the heme pocket. The magnitude of the conformational changes depends on the ligand in the order $CN^- > N_3^- > OH^-$, but the changes are of a very similar nature in each case. They mainly consist in some alterations at residues E5, E6, E7, and E8 as well as detectable movements at the Val E11 side group and Leu E15. There is evidence that the E-helix in CN-Mb has bent away from iron in the general area of residues from E5 to E8 [10,21].

Thus, our analysis has shown that the changes in the heme environment upon the modification most likely comprise some shifts of the distal His E7 and the adjacent region of the E-helix as well as shifts of the side groups of Phe B14, Phe CD1 and Phe CD4 which are incorporated in the hydrophobic cluster on the distal side of the heme.

These shifts appear to be due to the fact that modification of the N-terminal α-amino group disrupts, partly or totally, "salt bridges" at the N-end and this results in a changed position of the A-helix with respect to the GH region of the molecule. A mobili.y of the spin-label at His A10 did decrease by a factor about two followed the modification of Val NA1 by methylisothiocyanate [3].

So not only the electronic and ligand state of the heme complex in ferri Mb has been shown earlier to effect the ionic interactions and the conformation at the N-terminus [2,3] but the opposite influence also takes place. This example demonstrates electronic conformational interactions (ECI) in the protein molecule [23].

A structural basis of ECI appears to be in this case

synchronous movements of the A- and E-helices induced by some changes of the distal His E7 position upon the ligand substitution and a displacement of the E-helix, on the one hand, and by alteration of the stabilising ionic interactions at the N-end of Mb and a movement of the A-helix, on the other, caused by pH changing or complexing with other molecules. It follows from the Mb structure that the long antiparallel A- and E-helices like G- and H-helices are linked up by a large number of side group contacts that impart stability of their mutual position [1,24]. Moreover, from the structural point of view the whole ABCDE fragment may be regarded as a single domain (see fig. 5) and the pH- or ligand induced conformational transition in ferri-Mb might consist in a changed relative position of ABCDE and GH-fragments to each other [2,25]. In fact the conformational changes detected by X-ray, NMR and spin-label techniques occur in the contact area between ABCDE and GH-domains but not inside them. Conformational transition of this kind as one can see provides a relationship between and a mutual influence of, the state of the heme complex and the conformation at the N-terminus in the Mb and Mb-like structure.

The chemical modification of the α-amino group by isothiocyanate reagents simulates the abolishment its positive charge upon deprotonation and enables one to trace out changes in the heme region without altering the ambient pH and without the associated replacement of the sixth ligand. It is evident, however, that these effects are not quite adequate, since the alkaline titration of metMb up to deprotonation of the α-NH_2-group (pK = 7.8) also involves deionization of His residues, and consideration has therefore to be given to possible additional conformational alterations arising upon deprotonation of His residues with pK > 6, namely His G17 (pK = 6.43) and, especially, His C1 (pK = 7.7) [20]. The fact that the pK value of His C1 as determined from NMR titration data nearly coincides with the ionization pK of the α-amino group in metMb, provides support for the proposed scheme of heterotropic interactions in ferric Mb derivatives.

Acknowledgement

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