Studies on Interactions Between Metmyoglobin and Heparin

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Abstract. The complex formation between metmyoglobin and heparin was investigated by absorbance and fluorescence spectroscopy as well as differential scanning microcalorimetry. In acidic pH region, three distinct complexes detected by absorbance measurements are formed depending on pH and time of equilibration. The kinetics of the conformational transition of metmyoglobin-heparin complex equilibrated at neutral pH observed after pH change to acidic region comprises two steps. During the first step, characterized by rapid changes of the absorption spectra (approximately 5 minutes) as well as fluorescence intensities, reversible transition with $pK = 6.5 \pm 0.1$ occurs and the first type of the complex forms. Below pH 6.2 the transition with $pK = 5.7 \pm 0.1$ is observed and the second type of the complex is formed. During the second slow step, the third type of the complex formed after 30 minutes of equilibration is characterized by a spectrum corresponding to low-spin form without protein axial ligand bound. At neutral pH and 25 °C, the interaction between metMb and heparin only slightly alters absorption and fluorescence spectra. On the other hand, the formation of metMb-heparin complex is established from the decrease of the transition temperature from 80.4 ± 0.5 °C to 74.7 ± 0.5 °C. Moreover, the binding of heparin prevents the aggregation of the protein at isoelectric point resulting in a considerable increase in the reversibility of thermal denaturation.

Key words: Metmyoglobin — Polyanion binding — Optical spectroscopy — Fluorescence spectroscopy — Microcalorimetry

Abbreviations: metMb — metmyoglobin, Trp — tryptophan, DSC-differential scanning microcalorimetry, AsW — $(KAs_4W_{140})^{27-}$, SbW — $(NaSb_9W_{21}O_{86})^{18}$, HPR — heparin.

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Introduction

Myoglobin (Mb) is an oxygen-binding heme protein found in the heart and skeletal muscle tissue. The ferrous form of Mb is required for reversible oxygenation to occur. However the inactive metMb form is generated in the presence of oxidants, therefore a mechanism for reducing metMb is necessary. It has been shown that the metMb reduction system requires cyt b_5 as an electron transfer mediator. MetMb and cyt b_5 form a 1:1 complex through electrostatic interactions. Computer modeling reveals three positively charged residues of Lys of metMb and negatively charged carboxyl residues and heme propionate as contact points in the complex (Livingston et al. 1985). Absorption spectroscopic studies can provide insight into the molecular structure of these complexes, but difficulties arise when similar chromophoric groups of both proteins contribute to the measured spectra. The use of polyanions as models of natural basic redox partners appears advantageous because the polyanions contain no absorption bands in the visible region (Chottard et al. 1987, Hildebrandt 1990, Antalik et al. 1994, Sedlak 1997).

Chottard et al. (1992) have observed the binding of several polytungstates to metMb (as an example of neutral protein) with association constants and stoichiometry comparable to those of the cyt b_5-metMb complex.

Heparin is one of the strongest polyanions that occurs naturally in organisms (Jacques 1980, Fromm et al. 1995). The chain of the free molecule is hydrophilic and unfolded due to the electrostatic repulsion between the negatively charged groups. Heparin forms complexes with basic proteins by means of electrostatic interactions (Jacques 1980). Binding of heparin to these proteins induces conformational rearrangement and alters their activity (Antalik et al. 1992, Fromm et al. 1995). Comparing with polytungstates, the polyanion heparin lacks the great disadvantage of them, namely low stability in wide pH range.

In the present work we studied the complex formation between metMb and heparin in neutral and acidic pH regions. The resulting structural modifications upon heparin addition were investigated by optical absorption spectroscopy, fluorescence, and differential scanning microcalorimetry.

Materials and Methods

Heparin, sperm whale and horse skeletal muscle tissue metMb were obtained from Sigma Chemical Co. (St. Louis, USA). MetMb was used without further purification. Before every measurement, 18 μmol/l $K_3Fe(CN)_6$ was added to the sample to keep metMb fully oxidized.

HEPES and phosphate buffers were obtained from Serva (Heidelberg, Germany). The pH values were changed by addition of concentrated HCl, and were measured with a Sensorex glass electrode.

The concentration of metMb was determined spectrophotometrically (ε_{409}= 157 l/(mmol cm) for sperm whale and ε_{408}=188 l/(mmol cm) for horse heart) (Antonini and Brunori 1971) In titration experiments the sample was equilibrated prior to the measurements The equilibration of the sample was determined by monitoring the evolution of the absorption spectra Typical incubation times for complex formation were 2 hours, and 30 min for pH titration of the complex

Absorption measurements were made on a SHIMADZU UV 3000 spectrophotometer, and fluorescence measurements on a SHIMADZU-5000 The excitation wavelength for Trp was 290 nm The protein concentration was about 5 μmol/l Differential scanning calorimetric measurements were carried out using a highly sensitive DASM-4 microcalorimeter with a heating rate of 1°C/min and a temperature range from 18°C to 110°C All the experimental curves were calibrated to the base line obtained by heating of the buffer solution The protein concentration was about 90 μmol/l

The enthalpies of denaturation were evaluated from the experimental curves of the heat absorption according to the following equations

ΔH_{cal} was determined from the thermal effect Q_t of denaturation by relating the area of the heat absorption peak to the area of the calibration peak

The van't Hoff enthalpy was calculated from the equation

$$\Delta H_{vH} = 4R(T_m)^2 C_{pmax}/Q_t$$

where ΔH_{vH} is the van't Hoff enthalpy, R is the gas constant, C_{pmax} is the height of the heat capacity curve for $T = T_m$ (Privalov and Khechinashvili 1974)

The pK values of transition were obtained from the change of the maximum position of absorption bands at 505 535 nm, from the change of the intensity of the band at 535 nm and from the change of the fraction of fluorescence intensity and optical absorption at 395 410 nm, where zero value corresponds to the native state and value 1 to the state when conformational changes caused by polyanion binding are fully saturated

Results

Spectral properties of heparin binding to metMb

Optical absorption

Upon stepwise addition of heparin to the solution of metMb at pH 6 2 after 30 min equilibration and after each addition of heparin the following changes were observed the Soret peak was shifted from 408 nm to 395 nm and its intensity decreased the bands at 505 and 635 nm were weakened and a band was formed at 535 nm (Fig 1*a*, *b*) No additional changes were observed above heparin concentration 1 5 mg/ml Since the molecular weight of heparin cannot be evaluated exactly due

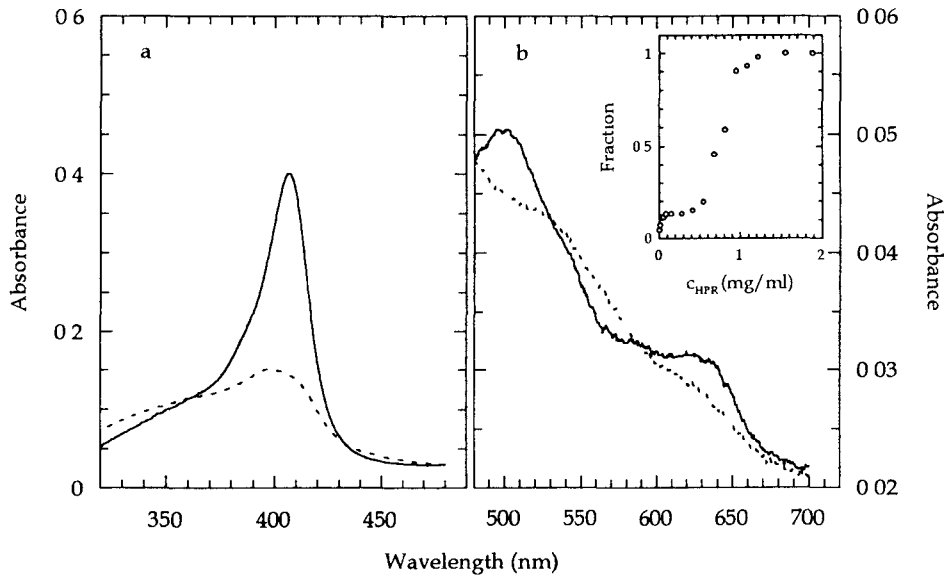

Figure 1. Absorption spectra of free sperm whale metMb (4 μmol/l) (solid line) and its complex with heparin (1 5 mg/ml) (dashed line) in Soret (*a*) and in 480 700 nm region (*b*) in 10 mmol/l HEPES buffer solution pH 6 2 Inset in *b* the plot of the complex metMb-heparin fraction detected by absorbance change at 408 440 nm vs heparin concentration Value 0 of the optical absorbance fraction corresponds to the native state and value 1 to the state when conformational changes caused by heparin binding are fully saturated

to the various lengths of the chain and the molar association constants could not be precisely determined, the complex formation was quantitatively characterized only in terms of gram per milliliter As can be seen from Fig 1*b* (inset), the complex formation between metMb and heparin is not a simple process Only small changes of the Soret absorbance change appear up to heparin concentration of 0 4 mg/ml Any subsequent increase of heparin concentration results in an abrupt increase of the Soret absorbance change, which is fully saturated at 1 5 mg/ml

At pH 7 3, the spectrum of metMb in the presence of saturation amounts of heparin after 2 hours of equilibration (Fig 2 solid line) was practically identical to that observed for native metMb Only the intensity of the Soret band slightly decreased (by about 8% not shown) It should be pointed out that the binding of heparin had no overall effect on metMb structure in the heme moiety at neutral pH This form of the complex should be classified as type N Fig 2 also shows the spectra obtained upon changing the pH from 7 3 to 6 7 and 6 2 after 30 minutes of equilibration until no additional changes of absorbance occurred The intensities

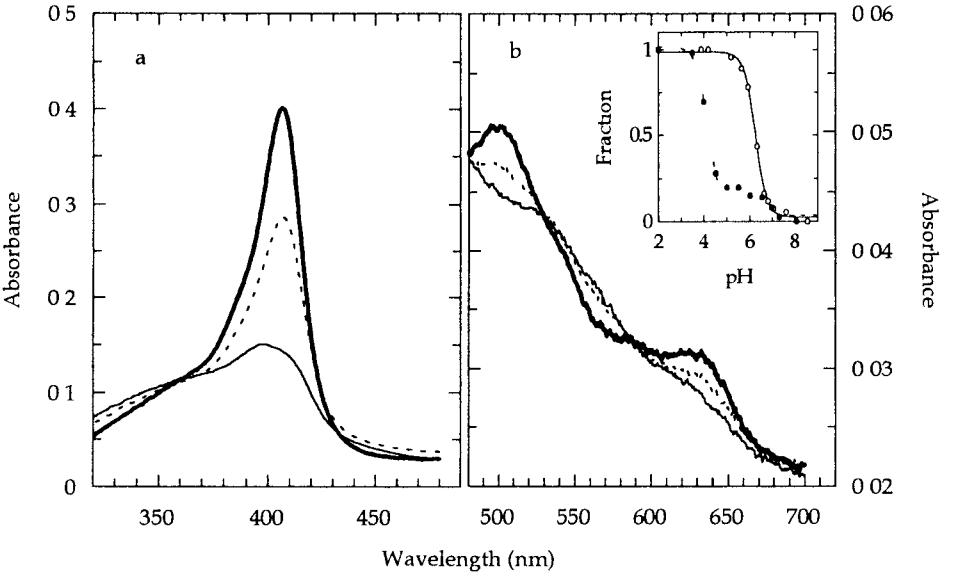

Figure 2. Influence of pH on the visible spectrum of sperm whale metMb-heparin complex in Soret (*a*) and in 480–700 nm region (*b*) in 10 mmol/l HEPES pH 7.3 (bold solid line) 6.7 (dashed line) 6.2 (solid line) Protein concentration 4 μmol/l heparin concentration 1.5 mg/ml. The spectra were obtained by changing pH from 7.3 to 6.7 and 6.2 after 30 minutes of equilibration until no additional changes of absorbance occurred. Inset in *b* acid denaturation curves of free sperm whale metMb (●) and metMb heparin complex (○) at the same conditions. Value 0 of the optical absorbance fraction corresponds to the native state and value 1 to the state when conformational changes caused by pH change are fully saturated

of the bands decreased with the lowering pH. The bands at 505 nm and 635 nm typical of high-spin form decreased, a band occurred at 535 nm and the Soret band slightly shifted from 408 nm to 395 nm. Even though the decreases of several bands could indicate the formation of low-spin species, the decrease of the intensity and the blue shift of the Soret band apparently reflected a partial exposure of the heme from the cavity, when the protein axial ligand was not yet bound to the heme. This partially low-spin form should be classified as type H. The acid-unfolding profiles of metMb and the metMb-heparin complex shown in Fig. 2*b* were obtained from the absorption spectra recorded in the Soret region. The pK constant of native metMb is 4.1 ± 0.1. In the presence of heparin the transition was shifted to a higher pH value $pA = 6.2 \pm 0.1$. It was not possible to convert the H form to N form by changing pH from acidic to neutral region.

The kinetics of the conformational transition of the metMb-heparin complex

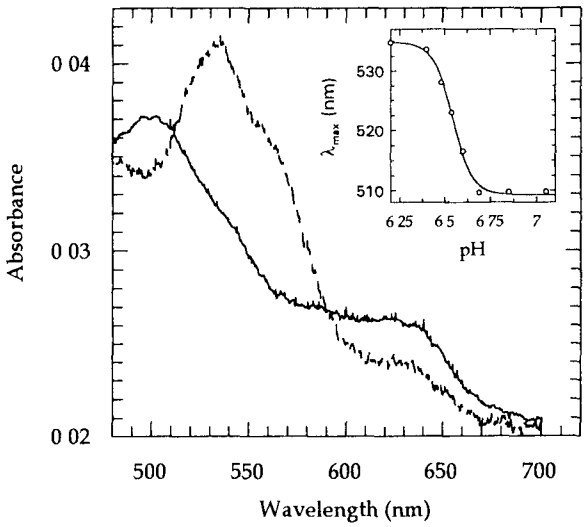

Figure 3. Absorption spectrum of sperm whale metMb-heparin complex in 10 mmol/l HEPES pH 7 3 (solid line), 6 2 (dashed line) obtained after 5 minutes upon pH change from 7 3 to 6 2 Protein concentration 3 μmol/l, heparin concentration 1 5 mg/ml Inset Dependence of the absorbance maximum position in 505 535 nm region on pH

equilibrated at pH 7 3 observed after pH change to acidic region comprised at least two steps (not shown) The first step was characterized by very rapid changes (approximately 5 min) in spectra The second step was a slow relaxation of the metMb heparin complex to unliganded heme H form (Fig 2) Whereas in the Soret region the absorbance only decreased with the decreasing pH, significant shifts of maxima positions were observed within the 500 700 nm region Fig 3 shows the absorption spectrum of the metMb-heparin complex at pH 7 3 and the spectrum obtained after 5 minutes upon pH change from 7 3 to 6 2 The band at 505 nm shifted to 535 nm and its intensity increased, the shoulder at 545 nm shifted to 565 nm and the intensity of the band at 635 nm decreased This form of the metMb heparin complex is classified as type I From the plot of the shift of the maximum position at λ_{max} region from 505 to 535 nm vs pH we obtained the value of $pK = 6\ 5 \pm 0\ 1$ of the transition between N and I form (Fig 3, inset) A decrease of pH to below 6 2 led to an additional shift of the band at 535 nm to 520 nm and an increase of the 635 nm band (not shown) From the plot of the absorbance of the band at 535 nm we established the pK constant for the transition between I and II form $pK = 5\ 7$ (Fig 4) Fig 5 shows the spectra of metMb-heparin complex at pH 4 5 and free metMb at pH 2 The form of the complex present at pH 4 5 should be classified as type II (Fig 5, solid line) The structure of the visible spectrum in

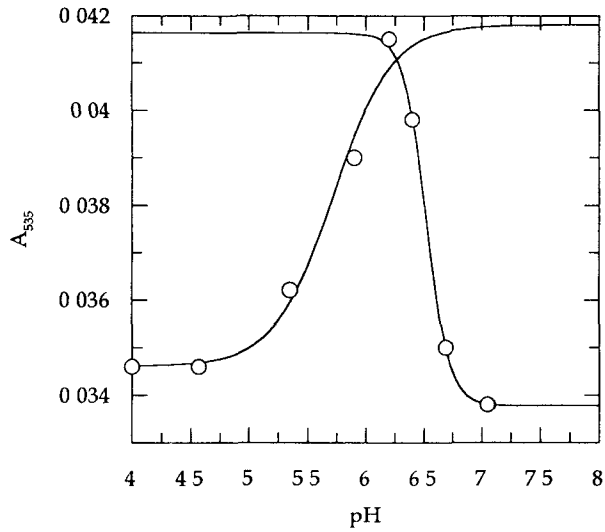

Figure 4. Plot of absorbance of the band at 535 nm for sperm whale metMb-heparin complex vs pH in 10 mmol/l HEPES, protein concentration 3 μmol/l, heparin concentration 1 5 mg/ml

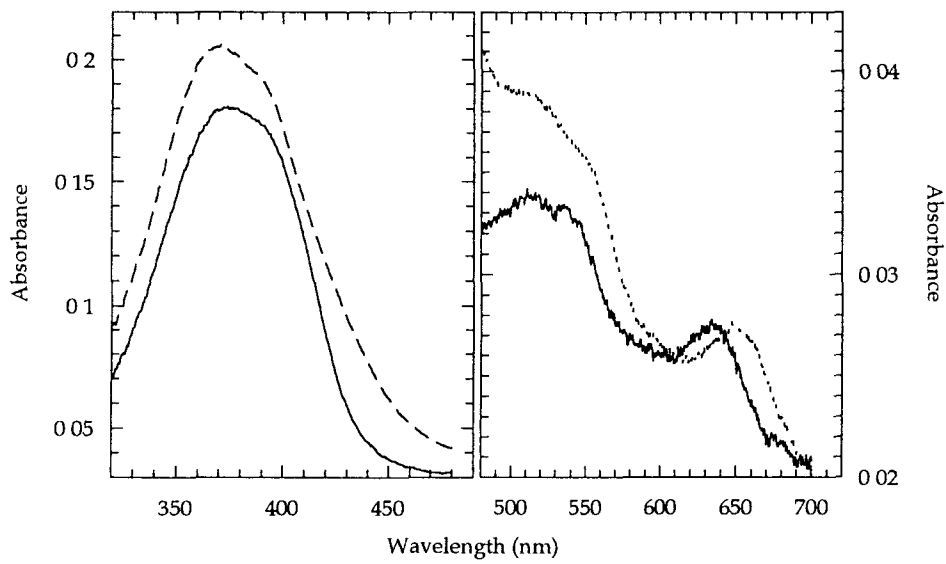

Figure 5. Absorption spectra of free sperm whale metMb (4 μmol/l) in pH 2 (dashed line) and metMb-heparin (1 5 mg/ml) complex at pH 4 5 (solid line) in 10 mmol/l HEPES buffer solution obtained after 30 minutes of equilibration

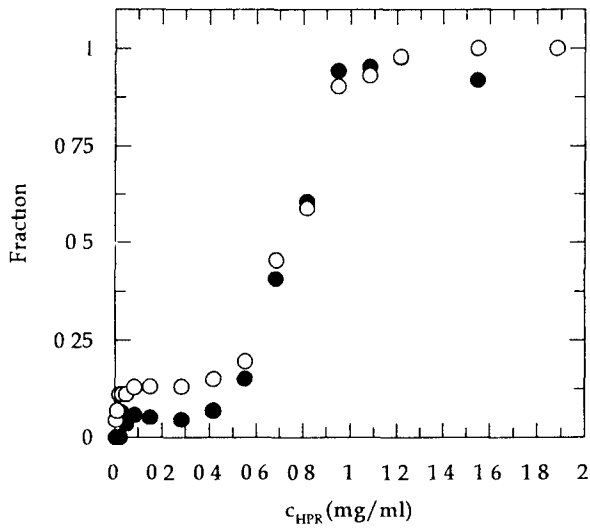

Figure 6. Dependence of normalized fluorescence emission at 350 nm (●) (excitation wavelength λ_{exc} = 290 nm) and normalized difference in absorption spectrum at 408 440 nm (○) for sperm whale metMb (4 μmol/l) on heparin concentration (c_{HPR}) in 10 mmol/l HEPES pH 6 2

Soret region resembles the spectrum of acid- denatured metMb at pH 2 the slight differences of the position of bands at 635 nm and absorbance at 535 nm indicate that the weak influence of heparin on metMb is still present at this pH and the complex is not broken at this pH While in the case of H type the increase of pH did not lead to structure renewal in the heme region, it is possible to convert type I to type N as well as type II to I by changing pH immediately after complex II has formed

Fluorescence

Two tryptophanyl residues Trp-7 and Trp-14 are located in the N-terminal region (Takano 1977) In the case of intact metMb, the intrinsic fluorescence of both tryptophans is partially quenched due to the energy transfer to the heme group (Hochstrasser and Negus 1984) The efficiency of the energy transfer depends on the distance and relative orientation between the heme group and the tryptophan molecules Hence Trp fluorescence seems to be a suitable marker of structural changes in the N-terminal region and the heme pocket Native metMb has a fluorescence emission maximum near 340 nm in response to excitation at 290 nm, the maximum of the fluorescence intensity of the protein corresponds to 10% of the fluorescence intensity of free Trp in the same concentration

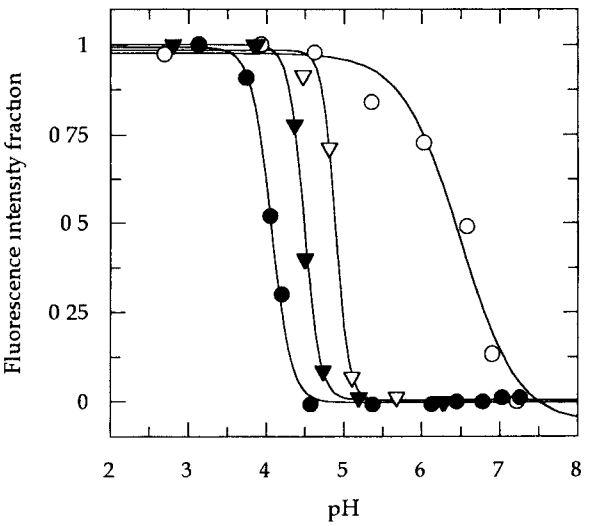

Figure 7. pH dependence of normalized fluorescence emission at 350 nm (excitation wavelength λ_{exc} = 290 nm) for free sperm whale metMb (● 10 mmol/l HEPES ▼ 10 mmol/l HEPES + 0.5 mol/l NaCl) and metMb heparin complex (○ 10 mmol/l HEPES ▽ 10 mmol/l HEPES + 0.5 mol/l NaCl) Protein concentration 4 μmol/l, heparin concentration 1.5 mg/ml

The change in fluorescence intensity versus heparin concentration at pH 6.2 is shown in Fig. 6. In the presence of increasing amounts of heparin the fluorescence intensity increases and the maximum of the emission spectrum is red shifted about 10 nm, which suggests that heparin binding results in the exposing of Trps to more polar environments than in native metMb. This Figure is a plot of the fraction of fluorescence intensity and optical absorption, where zero value corresponds to the native state and value 1 to the state when conformational changes caused by heparin binding are fully saturated. Although both curves are similar (Fig. 6) shorter time is needed to reach equilibrium in fluorescence measurements. As a result of comparison of both curves it may be concluded that perturbation due to heparin binding is not localised but is sequentially distributed from the surface throughout globule. In other words, the binding of heparin comprises cooperatively the entire protein molecule.

The relative fluorescent intensity of Mb and of the complex with heparin as a function of pH and ionic strength is shown in Fig. 7. After initial rapid equilibration, no additional changes of fluorescence intensity were observed. The transition with $pK = 4.1 \pm 0.1$ was typical for free metMb in 10 mmol/l HEPES. In the case of the complex the pK value was shifted to 6.5 ± 0.1. This pK value agrees with that

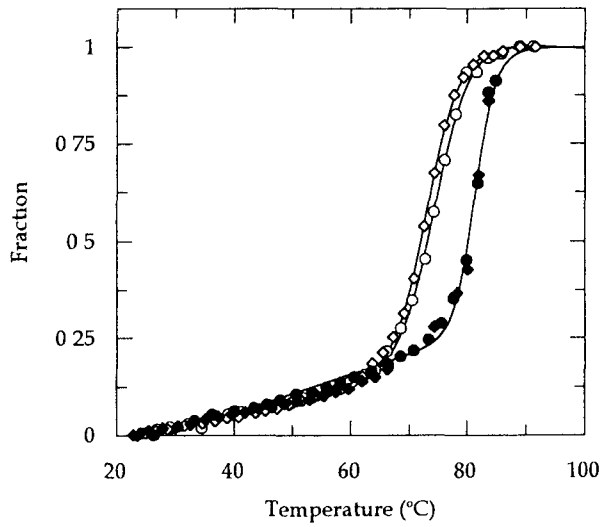

Figure 8. Temperature dependence curves of normalized difference in absorption spectrum at 408–440 nm for free (●) and complexed (○) sperm whale metMb as well as free (♦) and complexed (◊) horse skeletal muscle metMb in 10 mmol/l phosphate buffer solution pH 7 4 Protein concentration 4 μmol/l, heparin concentration 1 5 mg/ml

obtained from absorption measurements for the rapid process However the pK value of the transition established from absorbance spectra at lower pH was not obtained by the fluorescence method In the presence of 0 5 mol/l NaCl the pK constant of the complex decreased to 4 8±0 1 and approached the value of 4 4±0 1 for native metMb

Thermal stability of the heparin-metMb complex

Fig 8 shows the temperature dependence curves of the optical absorbance change at the Soret region of native sperm whale metMb and horse skeletal muscle metMb and their complexes with heparin in 10 mmol/l phosphate buffer, pH 7 4 It can be seen that similar transition midpoints were obtained for both sperm whale and horse skeletal muscle myoglobin The T_m value was 82 4 ± 0 5°C and van't Hoff enthalpy ΔH_{vH} was 437±30 kJ/mol for native metMb Addition of heparin altered the denaturation properties of the protein The transition temperature decreased to 73 3±0 5°C and the van t Hoff enthalpy to 349±30 kJ/mol The denaturation was only partially reversible probably due to exposing of the heme from heme pocket Thus in the process of heat denaturation, the presence of heparin in the solution slightly decreased the thermal stability of metMb

For the precise thermodynamic analysis of heat denaturation we used the

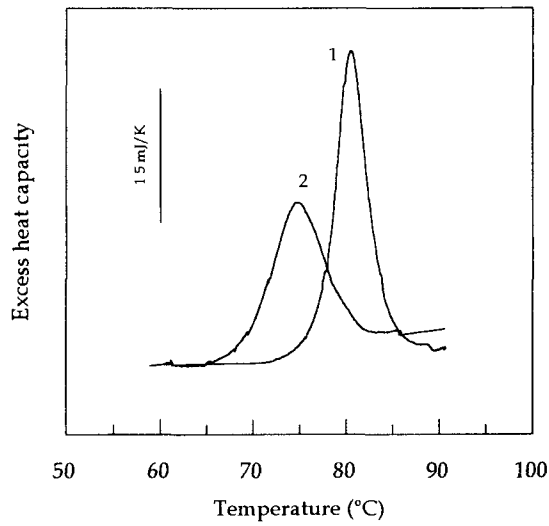

Figure 9. DSC-thermograms of free horse skeletal muscle metMb (curve 1) and metMb-heparin complex (curve 2) in 10 mmol/l phosphate buffer solution pH 7.4. Protein concentration 90 μmol/l, heparin concentration 3 mg/ml

calorimetric method. One of its greatest advantages compared to other methods is that not only calorimetric, but also van't Hoff enthalpy of denaturation can be obtained from the same experimental curve of heat absorption.

Fig. 9 shows the DSC thermograms for native metMb and for the complex with heparin in 10 mmol/l phosphate buffer solution at pH 7.3. The thermal transition of pure metMb was characterized by a single peak at 80.4 ± 0.5°C, calorimetric enthalpy $\Delta H_{cal} = 364 \pm 30$ kJ/mol and effective enthalpy $\Delta H_{vH} = 893 \pm 30$ kJ/mol. The ratio of ΔH_{vH} to ΔH_{cal} was 2.44. In the presence of saturation amount of heparin, the transition temperature is shifted to 74.7 ± 0.5°C and the values of the enthalpies decreased to $\Delta H_{cal} = 282 \pm 30$ kJ/mol and $\Delta H_{vH} = 590 \pm 30$ kJ/mol respectively. The ΔH_{vH} to ΔH_{cal} ratio was 2.0. The reversibility of heat denaturation of native metMb was only 10% due to the aggregation at this pH, the transition of the complex was 75% reversible.

The observed heat effect T_m and the shape of the melting curve detected by DSC did not depend on the heating rate (0.5–2°C/min), whereas heat denaturation monitored in heme region by Soret absorbance change was slightly rate-dependent. Therefore, there was a difference in van't Hoff enthalpies obtained by the calorimetric and the spectroscopic methods.

Discussion

In this paper we have shown the complex formation between metMb and polyanion heparin not only in acidic but also in neutral pH region. Even though the addition of heparin to metMb solution at neutral pH left the spectrophotometric parameters practically unchanged, the formation of the N form complex was detected from its influence on thermal stability. The presence of the complex in physiological conditions is of importance in the case of complex formation between metMb and its natural redox partner, cyt b_5. Mauk and Mauk (1982) have reported that no difference spectrum was elicited upon mixing of these proteins. We could show that even if heparin binding did not affect the visible spectrum at room temperature, it significantly decreased the transition temperature of heat denaturation of metMb at neutral pH.

It is generally known that in the pH range from 5.5 to 9 (conditions near to the isoelectric point) myoglobin has a strong tendency to aggregate and precipitate in the process of heating. Regarding this fact, we observed low reversibility (only 10%) of thermal denaturation of native metMb as a consequence of aggregation. However, the complex formation between metMb and heparin was found to partially prevent aggregation, resulting in a sharp transition with 75% reversibility and a slight decrease of the van't Hoff enthalpy and calorimetric enthalpy ratio from 2.4 to 2 at pH 7. The chain of heparin is unfolded due to the repulsion of the negative charged groups. Its interaction with metMb results in a lowering of the ability of the protein to form aggregates, probably due to the steric hindrance and repulsion of charged groups on the heparin chain and metMb molecule. Bagelova et al. (1994) have studied heparin binding to the basic protein cytochrome *c* (isoelectric point 10.3). In the presence of saturation amount of heparin, a more substantial decrease of transition temperature of cytochrome *c* by about 20°C (from 84.1°C to 61.4°C) has been observed at pH 7. A 63% reversibility of heat denaturation was achieved, and van't Hoff and calorimetric enthalpy ratio was 1.22. As metMb is neutral whereas cytochrome *c* is a basic protein, the lower decrease of transition temperature of metMb by about 6°C (from 80.4°C to 74.5°C) is probably due to the lower content of charged groups at neutral pH. However, the spatially displaced charged groups on the surface of the metMb molecule form the positively charged regions. These charged areas enable electrostatic binding with negatively charged groups on the heparin chain.

Different forms of metMb-heparin complex are observed in acidic pH region. Upon a rapid pH change, the whole molecule gets rearranged as could be deduced from the coincidence of pK constants (pK = 6.5) obtained from the absorption spectra of heme region and the fluorescence spectra of tryptophanyl residues located at a distance from the heme plane. This form of the complex is marked type I. A decrease in pH induced additional rearrangement in heme region and transition

from type I to II with pK constant 5 7, which could only be seen from absorption spectra After the initial global change, the absorption spectra of the heme region developed and the H type metMb-heparin complex was formed In this form the heme is partially exposed from the cavity and the axial protein ligand is not bound to the heme This conformational change of the heme region occurs slowly and is not seen in the Trp region The high content of van der Waals contacts of heme with the tightly packed nonpolar groups in the heme pocket is probably responsible for the slow rearrangement of groups in the heme region (Takano 1977) A similar behaviour is observed for free metMb after pH titration Faster changes are noticed for Trp fluorescence than heme optical absorbance due to the greater accessibility of Trp residues from the surface The transition between N form and H form is characterized by the apparent pK constant of 6 2 In contrast to the rapid equilibrated forms I and II which can be to converted to N type by reversing the pH value, no conversion of the H type complex to N is observed

Livingston et al (1985) have shown that cyt b_5-metMb complex is formed when protein concentrations are equal and the association appears to be strongest at pH 4-6 There is a physiological advantage of Mb-cyt b_5 binding at low pH because anaerobiosis and lactate production results in the lowering of sarcoplasmic pH from 7 1 to 6 7 (Ackerman et al 1980) This partially resembles our finding that evident complex is present within this pH range Even thought the pK constant of the transition for metMb-cyt b_5 complex has not been established, the pK constants for H and I metMb-heparin complex (6 2 6 5 resp) coincide with the fact that the pH optimum for enzymatic reduction of metMb is 6 5 (Livingston et al 1985) Chottard et al (1992) have studied the binding of several heteropolytungstates to metMb Different from our measurements they studied only equilibrated complexes and did not analyse processes occuring before the equilibrium The value of the pK constant of irreversible transition for slow equilibrated H type of the complex obtained in the present work partially coincides with the pK constant obtained by the above authors for metMb-SbW complex (6 5) and differs from pK = 7 4 for metMb-AsW complex which has stronger influence on metMb structure than heparin, probably due to the rigid arrangement and a higher content of charged groups in the interaction space

It is apparent that negatively charged partners bind more readily in acidic than in neutral pH region In its native conformation at pH 7 the polypeptide chain of Mb coils into a spherical shape This compact tertiary structure is stabilised through electrostatically interacting intramolecular ion pairs, hydrogen bonds and hydrophobic interactions (Bagelova et al 1994) A decrease of pH results in the neutralisation of negative charges on the carboxylic groups and the unveiling of the counteracting positive charges on the amino groups which disrupts the ion pairs and causes the protein to unfold (Ghelis and Yon 1992) The relaxed Mb chain after denaturation exposes other buried chargeable sites, such as the internal His residues

for protonation Hence the strong negative charge of the heparin chain favours the uptake of H^+ ions and the unfolding of Mb at higher pH values than native protein as could be deduced from the increase of the pK constant of transition from 4 1±0 1 for native metMb to higher values for all three types of the complexes observed

The properties of the formed complexes also depend on the ionic strength as is expected for electrostatic binding The electrostatic nature of the interaction between met Mb and heparin coincide with the fact that the formation of metMb-cyt b_5 complex is assumed to be realised by interactions between three positively charged Lys of metMb with negatively charged carboxylate groups

As follows from previous results the polyanion heparin appears to be a suitable model for the study of the properties of metMb in complexes with systems which contain locally distributed negatively charged groups such as cyt b_5 membranes chaperons, glycosidic proteins, not only in limited pH range as in the case of polytungstates but in a wide range of pH conditions

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