Association of iron-protoporphyrin-IX (hemin) with myosins

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Addition of myosins isolated from guinea pig heart and rabbit skeletal muscle to hemin solutions resulted in the appearance of new absorption spectra indicating association of hemin and the myosins. Binding stoichiometry based on absorption changes was found to be two hemin sites per myosin molecule. The binding constants calculated from quenching of the intrinsic fluorescence of the myosins by hemin are $K_a = 7\ (\pm 2)\ 10^6\ M^{-1}$ for skeletal muscle myosin, and $K_a = 3\ (\pm 1) \times 10^7\ M^{-1}$ for heart muscle myosin. Based on these findings, myosins are suggested as potential transporters of free hemin between cell organelles.

Myosin; Cytoskeleton; Hemin

1. INTRODUCTION

Iron-protoporphyrin-IX (hemin) is an active metabolite in a variety of essential, life-compatible processes. Being extremely active it is also toxic, and therefore cannot be available in free form: once synthesized in the mitochondria, hemin must be translocated to its target apoproteins to form hemoproteins. The carriers directing this hemin transport between cell organelles are believed to be heme binding proteins. While several cytosolic proteins have been suggested as carriers [1,2] none have been proven to date [3], and the question remains whether hemin carriers are to be found among the randomly moving cytosolic cell proteins or the less soluble, structurally organized cell proteins which are alternative and probably more efficient vehicles for hemin transportation.

Being a hydrophobic molecule, hemin will either bind to specific hydrophobic pockets in proteins – as in the case of hemoglobin – or attach in a weaker manner to available hydrophobic segments in semi-hydrophobic proteins – like in the serum heme transporting proteins hemopexin and albumin [4,5]. All structural proteins are semi-hydrophobic and therefore theoretically capable of providing binding sites for hemin. Indeed, basic myelin protein, the structural protein of the nerve fiber sheath, was reported to bind hemin at a defined site [6]; and the main components of red cell membrane cytoskeleton proteins, spectrin, protein 4.1 and actin, were shown to associate with hemin [7–9]. Since cell cytoskeletons are protein networks which traverse the cell, heme-binding sites on filamentous proteins may serve in heme transportation to various cell targets.

Myosin is an abundant structural protein of the cytoskeleton and the contractile protein systems in all vertebrates. The study presented here analyzes the possibility that myosin might associate with hemin and thus serve as its carrier. Myosins from the two striated muscle sources, skeletal muscle myosin (SMM) and cardiac muscle myosin (CMM), were studied since the thick filaments of muscle cells are the main venue of vertebrate myosins.

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2. EXPERIMENTAL

2.1. *Proteins*

Myosin isolated from rabbit [10] was a kind gift from Prof. A. Muhirad, Hadassah Medical School, Jerusalem. Guinea pig myosin was isolated from heart ventricles by described methods [11]. Myosins and their reaction mixtures were kept in 0.1 M pyrophosphate buffer solutions at pH 7.2 throughout the entire study. The concentration of myosin stock solutions was determined by the Lowry method [12], and 500 K was taken as the molecular weight of myosin.

Sperm-whale myoglobin (Sigma product) in the Met-form was solubilized in pyrophosphate buffer pH 7.2 and centrifuged at 50000 *g* for 10 min to remove any particulate matter. Concentration of myoglobin was determined spectrophotometrically using an EmM at 556 of 11.8 for the oxidized, ferri-form.

2.2. *Hemin*

80% DMSO stock solutions of hemin (Sigma) were used, and care was taken not to exceed 3% of DMSO in the protein-containing solutions to avoid denaturation. Stock solutions were centrifuged for 20 min at 50000 × *g* to remove unsoluble material. Hemin concentration was measured spectrophotometrically using an EmM of 170 at 403 nm in 80% DMSO. All other chemicals purchased from Sigma, were of analytical grade.

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2.3. *Instruments*

Spectrophotometric measurements were performed on a Shimadzu UV-160, an UV-visible light recording spectrophotometer. For fluorescence measurements, a Hitachi-Perkin Elmer 44B spectrofluorimeter was used.

3. RESULTS AND DISCUSSION

3.1. *Spectral changes of hemin in the presence of myosins*

The addition of myosins to freshly prepared hemin solution resulted in spectral changes of the hemin Soret band. Spectra were recorded at low hemin concentrations, where it exists mainly as a monomer in the water solution. Fig. 1 compares the spectral properties of hemin in buffer alone and in the presence of CMM and shows that addition of myosin resulted in (i) an increased extinction coefficient of a red shifted Soret band, and (ii) formation of two new absorption bands in the visible and near UV regions. These spectral changes are indicative of hemin binding to the protein. The hemin spectra in the presence of SMM and CMM are similar but not identical (Figs 1 and 2): the Soret maximum of hemin is red shifted in the SMM-hemin complex and the absorption extinction coefficient is smaller than that of the CMM-hemin complex. In addition, the near UV band is not completely resolved from the Soret band in the SMM complex, due perhaps to overlap of the two bands or increased contribution of free hemin. The latter possibility is consistent with our findings reported below that SMM has lower affinity for hemin than CMM. The spectral differences between myosins from cardiac and skeletal muscle may be attributed to the different myosin isoproteins in each of the muscle sources [13]. Throughout this study minor variations also appeared in the spectra of different preparations from the same muscle source which may have resulted from variations in weights of the isomyosins existing in each muscle source as well [13]. The Soret peak of hemin was at 410–414 nm in the presence of CMM, and at 418–421 nm range in the presence of SMM. The near UV peak, which was observed clearly only in CMM myosin, was at 330 nm, and the visible band of the two hemo-myosins was at 540–560 nm. These spectroscopic features of 'hemo-myosins' from the two sources resemble the general characteristics of low spin iron heme in hemoproteins such as peroxidase, catalase and hemoglobin. The positions of the absorption bands of the hemoglobin low spin derivatives are: a Soret band at 418–425 nM, a near UV peak in the 345–360 nm range, and a visible band at 540–560 nM [14].

To determine the number of binding sites, hemin aliquots were added stepwise to the myosin solution and the absorption was recorded after each addition at two wavelengths: (i) 446 nm (chosen instead of the Soret peak to minimize the contribution of free hemin including from changes in the monomer-dimer

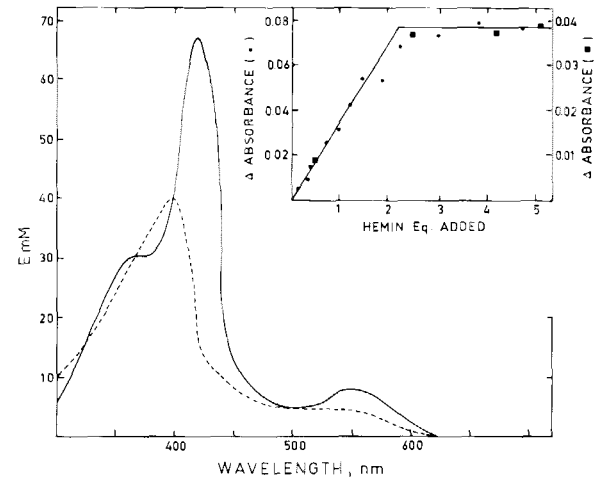

Fig. 1. Spectral characteristics of hemin binding to CMM myosin. The absorption of free and myosin bound hemin in the visible range is compared. In each case the spectrum was taken 30 min after addition of hemin and recorded at ambient temperature. Broken line, 1 μM hemin in 0.1 M pyrophosphate buffer pH 7.2; solid line, 1 μM hemin in 0.1 M pyrophosphate buffer pH 7.2 in the presence of 2.5 μM CMM myosin. Insert: Difference titration of hemin with myosin: 0.1 M pyrophosphate buffer pH 7.2 in the presence of 2.5 μM CMM. Shown is the absorbance of hemin in CMM containing solution vs hemin added to buffer only. (●) At 446 nm; (□) at 550 nm.

equilibrium), and (ii) 550 nm, the peak of the visible band. In the inserts of Figs 1 and 2 difference titrations of hemin added to buffer containing CMM or SMM respectively vs hemin in buffer alone are demonstrated. As seen, the intersection of the two straight line segments (sloped where additional extinction of the protein bound hemin contributes and parallel to the x coordinate where binding to protein stops), is at two hemin equivalents per myosin molecule. Since myosin has a longitudinal symmetry of two, we propose that each half molecule contains a single hemin. However, the exact location of the two sites remains to be determined. The two sites are spectroscopically equivalent and, in this regard at least, independent. It is interesting to note that spectrin, which has a similar molecular weight of about 500 K, binds many (~20) hemin molecules in what appears to be a hydrophobic non-specific manner [7]. Since in the case of myosin only a single hemin molecule binds to a protein unit of 250 K, myosin seems to provide a specific site for hemin. As specificity usually correlates with higher affinities, it was deemed of interest to compare binding affinities of hemin to myosins from different sources.

3.2. *Binding isotherms of hemin and myosin*

The affinities of myosins and hemin were measured using fluorescence methods, which permitted low protein concentrations providing equilibrium conditions. Binding of hemin to myosin was followed using the intrinsic (tryptophans) fluorescence of the protein. The addition of very low hemin concentrations to myosin

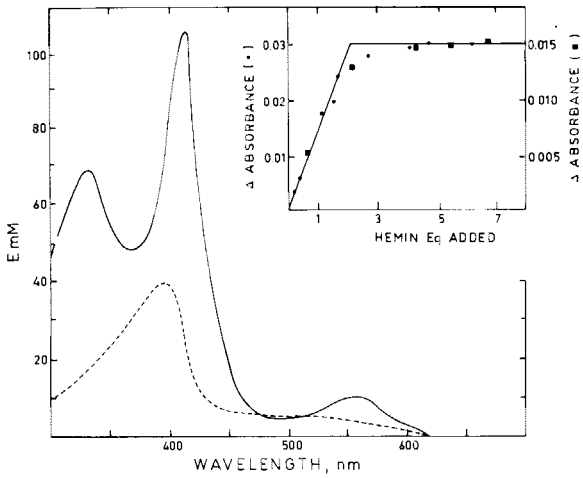

Fig. 2. Spectral characteristics of hemin binding to SMM myosin. The absorption of free and myosin bound hemin in the visible range is compared. In each case the spectrum was taken 30 min after addition of hemin and recorded at ambient temperature. Broken line, 1 μM hemin in 0.1 M pyrophosphate buffer, pH 7.2; solid line, 1 μM hemin in 0.1 M pyrophosphate buffer, pH 7.2, in the presence of 2.9 μM SMM myosin. Insert: Titration of hemin with myosin: 0.1 M pyrophosphate buffer, pH 7.2, in the presence of 3.5 μM. (●) At 446 nm; (□) at 550 nm. Difference titration of hemin with myosin: 0.1 M pyrophosphate buffer pH 7.2 in the presence of 2.5 μM SMM. Shown is the absorbance of hemin in SMM containing solution vs hemin added to buffer only.

solutions resulted in quenching of the fluorescence intensity. Since no trivial filter effect exists at the hemin concentrations used, the emission quenching was attributed to radiationless energy transfer from the excited tryptophan donors to the protein bound hemin acceptors. The fluorescence quenching curves of myosin in the presence of increasing hemin concentrations are shown in Figs 3 and 4 for CMM and SMM, respectively. The amount of fluorescence quenched at infinite hemin concentration was calculated for each myosin from double reciprocal plots of the fluorescence intensity and hemin concentration, (data not shown). It was found that 62% of the CMM and 71% of the SMM fluorescence intensity are hemin quenchable. Thus, fluorescence criteria, like spectral data, show the hemin sites in myosins from the two sources to be similar but not identical.

The fluorescence quenching data were translated into the fractional saturation of hemin sites ν by $\nu = \Delta F/\Delta F_\infty$, where ΔF represents the fluorescence intensity quenched at various hemin concentrations and ΔF_∞ at infinite hemin concentration. The concentration of free hemin (C_f) at each step of the titration was calculated by substracting bound from total hemin. The inserts of Figs 3 and 4 illustrate the binding data drawn as Hill plots for myosins from the two sources: the curves are linear slopes of one within experimental error. The binding affinities for the two myosins and hemin were calculated from the Hill plots as $K_a = 3(\pm 1) \times 10^7\ M^{-1}$ for CMM, and $K_a = 7(\pm 2) \times 10^6\ M^{-1}$ for SMM.

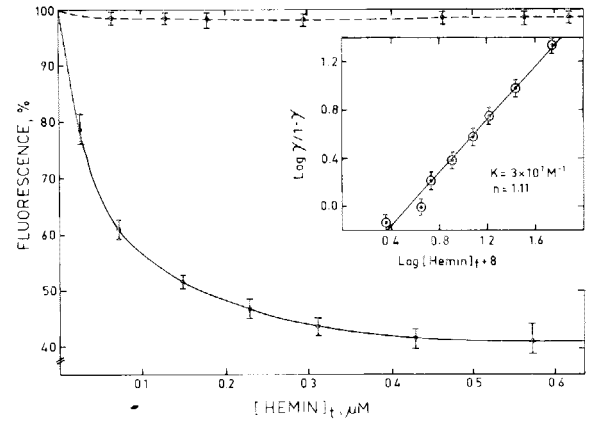

Fig. 3. Binding isotherm of hemin and CMM. Fluorescence data: excitation wavelength 280 nm and emission 342 nm. Temperature 37°C. Buffer 0.1 M pyrophosphate, pH 7.2, protein concentration 0.014 μM. Fluorescence intensity of the protein solution in the absence of hemin was set as 100%. Insert: a Hill plot of the binding curve in the main figure. Solid line, titration with free hemin; broken line, titration with myoglobin.

To determine whether variations in affinity resulted from differences in the organ or animal source, myosin was also isolated from the guinea pig skeletal muscle and titrated with hemin. The same binding constants (data not shown) were calculated for the guinea pig skeletal muscle myosin as for the rabbit skeletal muscle myosin. It is thus possible that in general cardiac myosins may have higher affinities for hemin than skeletal muscle myosins. The finding that hemin in myoglobin was unable to quench the fluorescence intensity of the myosins (Figs 3 and 4) indicates the failure of myoglobin to associate with myosins under our experimental conditions.

In summary, our results show that myosin from different sources can associate with hemin. The differences in myosin isoforms, as expressed in the

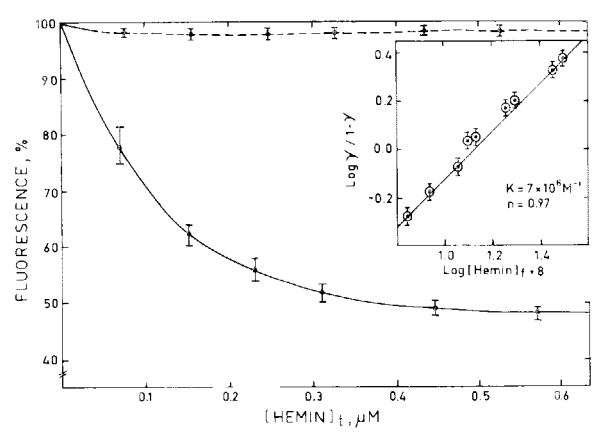

Fig. 4. Binding isotherm of hemin and SMM. Fluorescence data: excitation wavelength 280 nm and emission 342 nm. Temperature 37°C. Buffer 0.1 M pyrophosphate, pH 7.2, protein concentration 0.014 μM. Fluorescence intensity of the protein solution in the absence of hemin was set as 100%. Insert: a Hill plot of the binding curve in the main figure. Solid line, titration with free hemin, broken line, titration with myoglobin.

spectral characteristics and affinity towards hemin, demonstrate variability in the protein milieu of the hemin binding sites in myosins from various muscles. It is tempting to suggest that skeletal proteins in the cells might serve as vehicles directing hemin to desired locations in the cell. Affinity of hemin to myosins is higher than that of actin [9], which means that any hemin reaching filaments containing both proteins will transfer to the high affinity site on myosin. Thus, myosins might serve as last way stations of hemin prior to its final association with high affinity target apoproteins such as globins.

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