Effect of Guanidium Hydrochloride on the Stability of Horse Skeletal Muscle Myoglobin

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ABSTRACT: The stability of the three dimensional structure of horse skeletal muscle myoglobin was investigated using visible spectroscopy. Guanidium hydrochloride (GuHCl) of concentrations 0.4 – 0.8M have no observable effect on the three dimensional structure as indicated by monitoring the absorbance at 420nm. However, higher concentrations (1.0-3.0M) resulted in unfolding of the protein as indicated by the dip in absorption from 0.535 to 0.350.The requirement for high denaturant concentration to perturb the structure of myoglobin indicates the high stability of the protein. We conclude that high concentrations of the denaturant GuHCl, disrupts the 3-dimensional structure of myoglobin causing its unfolding, in a two- state process, due to weak binding to the protein, which can be studied spectrophotometrically

INTRODUCTION

Proteins carry out the most important tasks in living organisms. To do so, most proteins fold spontaneously into a well defined three – dimensional structure (Clarke and Waltho 1997, Johnson and Craig 1997, Ruddon and Bedows 1997; Ellis *et al*, 1998) which is required for specific interactions. But it was recently shown that several proteins do not assume stable three dimensional structures but are natively unfolded (Fink, 2005). The study of protein folding was initiated in 1910 by Henrietta Chick and C.J. Martins. They showed that the flocculation of a protein was composed of two distinct processes: precipitation from solution which was preceded by another process called denaturation, in which the protein became less soluble, lost its activity and became more chemically active. In mid 1920s, Anson and Mirksky, reported in Michael (1989) that denaturation was a reversible process. The hypothesis also suggested that denaturation was a two-state ("all-or-none") process, in which a fundamental molecular transition resulted in drastic changes in solubility, enzymatic activity and chemical reactivity. In 1929, Hsienwu hypothesized that denaturation was protein folding, a purely conformational change that resulted in the exposure of amino acid side chains to the solvent. In the early 1970s, Christian Anfinsen showed that the folding of ribonuclease A was fully reversible with no external cofactors required, thus verifying the "thermodynamic hypothesis" of protein folding that the folded state represents the global minimum of free energy for the protein (Anfinsen, 1973).

Proteins generally absorb light and emit radiation in the ultraviolet (UV) region of the spectrum. The absorption in the UV region is due to peptide groups, aromatic amino acids and to a small extent disulphide bonds (Schmidt, 2004). Absorption of light by proteins in the 230-300nm range is caused by aromatic side chains of tryptophan, tyrosine and phenylalanine. The spectrum of a protein is therefore dominated by contributions of tyrosine and tryptophan (Jones, 1997).

The spectral characteristic of a protein molecule depends upon the molecular environments and upon the mobilities of its chromophores (Schmidt, 2004). Spectroscopic measurements can be conducted in solution requiring only minute quantities of the protein, they are therefore useful for investigations of changes in the behaviour of a protein under different solvent conditions and also to compare the properties of related molecules, such as homologous or mutated forms of a protein (Yanon and Bovey, 1960). Importantly, spectroscopic methods are widely used to determine protein stability and to follow structural transitions such as unfolding and refolding under a variety of conditions (Pace *et al.*, 1989; Eftink, 1991; Creighton, 1993).

The folded conformation of a protein can be perturbed in various ways. Destabilizing agents such as urea or guanidium chloride and heat are widely used to cause protein unfolding (Creighton, 1993; Sykes *et al*., 1999; Schmidt, 2004).

Myglobin is a simple monomeric oxygen-binding protein found within muscle cells. Its crystal structure was the first to be elucidated and its reversible unfolding has been well studied (Schechter and Epstein, 1968; Puett 1973). It is one of the most extensively discussed proteins in biochemical literature because of its obvious historical and biological significance. Myoglobin absorbs light in the visible region (400nm) and undergoes a significant change in colour intensity upon denaturation (Sykes *et al*., 1999) thereby providing an opportunity for its stability to be studied using visible spectroscopy. With the advent of production of recombinant proteins, *in vitro* protein folding has assumed considerable importance (Stempfer *et al*., 1996). In this paper, we present report of myoglobin stability studies using Guanidium hydrochloride as denaturant.

MATERIALS AND METHODS

Myoglobin (M 0630) and Guanidine hydrochloride (G 3272) were both purchased from Sigma-Aldrich Company, Germany. The myoglobin was used without further purification. Disodium hydrogen orthophosphate and sodium dihydrogen orthophosphate were of analytical grades obtained from BDH Chemicals Limited, England.

Absorption spectrum of myoglobin

Two hundred milligrammes (200 mg) per ml solution of horse skeletal muscle myoglobin were prepared in 0.05M sodium phosphate buffer (pH 7.0) and two mls were transferred into a cuvette and absorption scanned from 300-600nm at interval of 20nm using a spectrophotometer (Model 6100, Jenway, England).

Stability study

For each reaction tube the concentration of myoglobin was held constant (0.2mg/ml) and the concentrations of guanidium hydrochloride (in the same buffer as myoglobin) were varied from 0.0 to 3.0M (in 0.2M increments). All samples were prepared in duplicate with buffer and GuHCl added first with gentle mixing and the protein added last. Samples were incubated for 30minutes at 25^0C before absorbance was measured at 420nm.

RESULTS AND DISCUSSION

The results of wavelength-absorption scanning of myoglobin are presented in Figure 1. Maximum absorption of light occurred at 420nm, the so-called Soret band. Minimum absorption was at 600nm. Absorption of light was maximal between 350 and 440nm.

Effect of the denaturant (GuHcl), on the conformation of myoglobin is presented in figure 2. As shown in the figure the proteins conformation was unperturbed when the denaturant's concentrations ranged from 0.4 to 0.8M with the absorbance value remaining at 0.535. With increasing concentrations of denaturant starting from 1.0M (beyond 0.8M) absorbance was drastically reduced to 0.384 which remained largely unchanged even when the concentration was gradually raised to 3.0M (indicated by the flat curve in Figure 2).

Myoglobin is a relatively small protein consisting of a single polypeptide chain. The architecture of the protein is arranged to permit reversible binding of oxygen to the haem prosthetic group, which lies buried in a hydrophobic pocket in the interior of the protein. The crystal structure of myoglobin was the first to be elucidated and its reversible unfolding well characterized (Kendrew, 1961).

Our study shows that myoglobin absorbs maximally at 420nm in the Soret region. Puett (1973) reported similar findings with ferrimyoglobin in phosphate buffer using circular dichroism in the ultraviolet region. The interaction of the haem with the protein portion of myoglobin results in the Soret band, a strong absorbance peak in the visible region between 409-420nm. Upon denaturation, the haem becomes exposed to the polar environment hence the decrease in absorbance (Sykes *et al*., 1999).

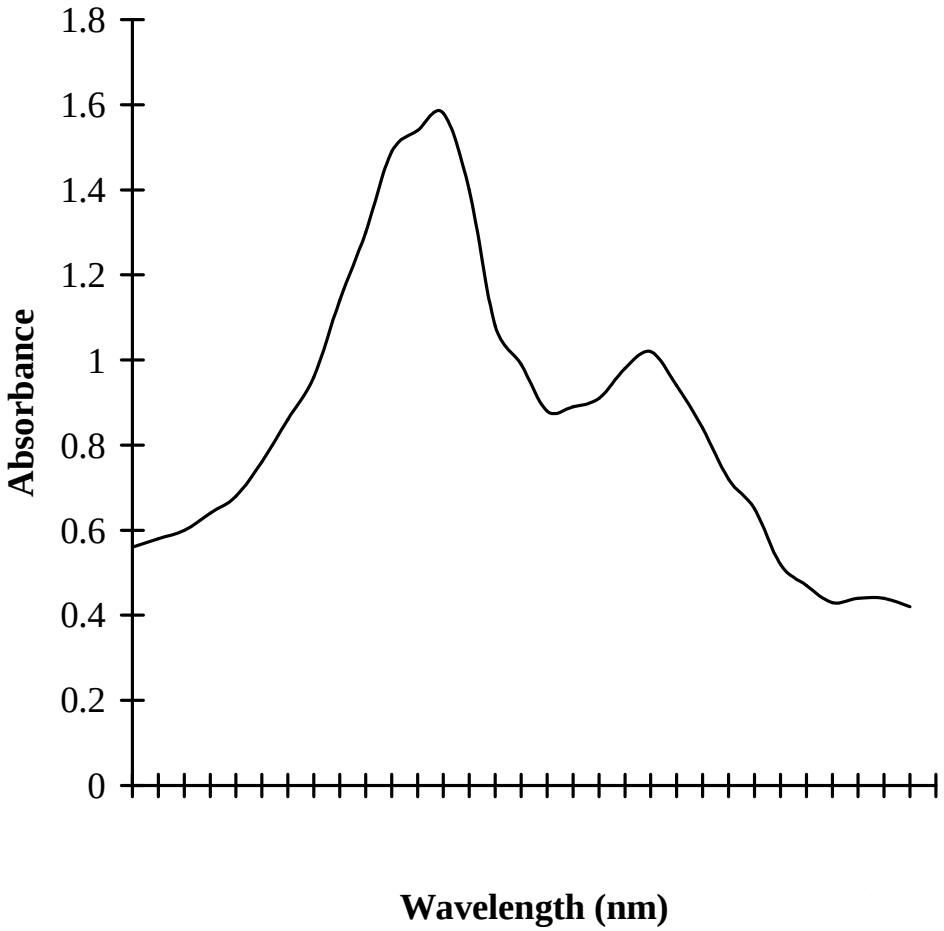

Figure 1: Absorbance Spectrum Of Horse Skeletal Muscle Myoglobin (native) In 0.1M Phosphate Buffer, pH 7.0

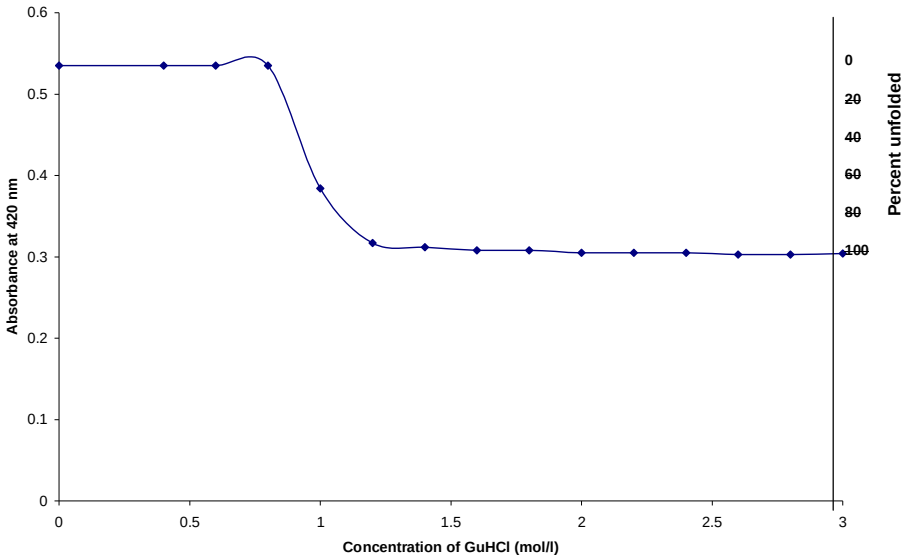

Figure 2: Denaturation profile of myoglobin in the presence of increasing concentrations of guanidium hydrochloride

The absorbance in the Soret region is independent of GuHCl concentration but affects only the ellipticity of the molecule (Puett 1973). This study explores the denaturation of myoglobin monitored by visible spectroscopy. Most reported studies of myoglobin stability employed fluorescence spectroscopy (Yanon and Bovey, 1960). Absorption of light by myoglobin in the visible region is due to tryptophan, phenylanine and tyrosine chromophores via transfer mechanism involving dipole-dipole interaction (King, 1989). Horse heart myoglobin possesses two tyrosine, two tryptophan and seven phenylalanine residues (Kendrew, 1963).

The folding of a protein is governed by several factors important among which are physical and chemical properties of the amino acid residues and the environment. The stability of the protein structure in solution (*in-vitro*) is usually measured by their resistance to denaturation in the presence of either heat or chemical denaturant (Schmidt, 2004). Other workers have reported stability studies on chymotrypsin (Kendrew, 1963) and ribonuclease A (Moore and Stein 1973) using guanidium ion as denaturant.

The drastic drop in absorbance from 0.535 to 0.384 as the myoglobin unfolded, as shown by our result, with no intermediate spectral events, lends support to the two- state model for myoglobin unfolding. The model assumes a two–state thermodynamic system with N representing the native state and U representing the fully unfolded state (Finn *et al*., 1992). Plotting the denaturation curve of the fraction of native or unfolded molecules versus the concentration of the denaturant will provide information for characterizing the transition between N and U and will also enable estimation of the conformational stability of myoglobin to be made in the absence of the denaturant so as to obtain a measure of the inherent propensity of the molecule to unfold under physiological conditions. Whether the two-state model will apply to larger protein molecules such as haemoglobin, pyruvate dehydrogenase etc remains to be investigated.

The guanidium ion increases the solubility of both polar and non polar amino acid side chains and thereby reduces the hydrophobic effect on protein stability (Nosaki and Tanford, 1970; Schellman, 1987; Creighton, 1993). High denaturant concentration can cause almost complete unfolding into a random polypeptide chain (Shortie, 1996). The spectral change from the native to unfolded state is large. The larger the spectral change accompanying the transition from the native to the denatured state, the greater the sensitivity of the method of its monitoring. The study indicates that high concentration of GuHCl is required to cause myoglobin to unfold. The requirement of high denaturant concentrations to effect conformational change in myoglobin indicates a great degree of stability of the protein, analogous to the case of sperm whale myoglobin (Schechter and Epstein, 1968). We conclude that high concentrations of the denaturant, GuHCl, disrupts the 3-dimensional structure of myoglobin causing its unfolding due to very weak binding to the protein, which can be studied spectrophotometrically in the visible region.

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