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Structural Basis of Heme Reactivity in Myoglobin and Leghemoglobin: Thermal Difference Spectra†

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ABSTRACT: Thermal perturbation difference spectra of sperm whale myoglobin (Mb) and soybean leghemoglobin a (Lb a) in the near-ultraviolet reveal similarities in the tryptophan environments of the two proteins. Of the two tryptophans in each protein, one has its indolyl NH group fully exposed to aqueous solvent, while the other behaves as if it were surrounded by motile but nonpolar residues with little access to water. These environments are not significantly altered by removal of the heme group. Assuming conformational homology, the helix-spacing role of Trp-A12 in Mb (Kendrew, J. C. (1962), *Brookhaven Symp. Biol. 15,* 216–228) may be taken over, in Lb a, by Trp-H8 which, though remote in linear sequence, would occupy a suitable spatial location. Thermal difference spectra in the Soret and visible regions of pure high-spin (fluoroferric) and pure low-spin (cyanoferric) complexes showed a red shift on cooling Mb complexes, reflecting a predominantly nonpolar environment around the heme, but a blue shift on cooling Lb complexes, reflecting a more solvent-exposed environment. Thermal difference spectra using rose bengal as a probe of the heme pockets in the two apoproteins supported these conclusions. Thermal difference spectra for the high-spin complexes of both Mb and Lb are slightly larger in magnitude than in the low-spin complexes. This may reflect a more flexible heme pocket in the high-spin state, as suggested by recent circular dichroic results. A structural basis for the high oxygen affinity of Lb compared with Mb is proposed, based upon the observed differences in polarity and flexibility of the heme pocket and in amino acid substitutions.

Mammalian myoglobins and plant leghemoglobins are the contemporary products of an evolutionary divergence occurring some 1200 million years ago. The observation of homologies in sequence (e.g., Dayhoff, 1972) and folding (Vainshtein et al, 1975) is all the more remarkable. The work described here forms part of studies designed to compare the structures of mammalian and plant hemoglobins with respect to folding, heme, and chromophore environment and their relationship to oxygen and ligand-binding effects. The technique of thermal perturbation difference spectroscopy is applied.

Thermal perturbation difference spectroscopy of proteins was developed by three groups (Bello, 1969a; Cane, 1969; Smith, 1970; Leach and Smith, 1972). In principle, it is analogous to the solvent perturbation difference spectral method introduced by Herskovits and Laskowski (1960), except that the perturbant employed is a small change in temperature rather than a change in solvent. Both techniques have been used to determine the degree of exposure of aromatic amino acids (mainly tyrosine and tryptophan) in native proteins. There are some advantages of thermal over solvent perturbation for such purposes. (1) The perturbation (temperature difference) can be varied continuously on the same sample. (2) If the sample temperature is cooled by say 10 °C, such a perturbation is less likely to cause conformational changes in the protein than is a change from aqueous solvent to 20–40% organic solvent. (3) The baseline can be internally checked by recording a difference spectrum when both sample and refer-

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ence cell are at 25 °C before and after the experiment. Similarly, reversibility can be checked by recording difference spectra as the temperature is decreased below or increased back to 25 °C. (4) Conformational or other changes in the protein within the temperature range used can be detected, since these usually will cause the size of the difference spectrum to be nonlinear with temperature difference. (5) The parameter measured by thermal perturbation is exposure to water and this is a biologically more relevant parameter than the interaction of chromophores with organic solvents.

Thermal difference spectra of proteins are thought to originate from thermal changes in the interactions between chromophores and aqueous solvent; the nature of these interactions and their expected temperature dependences have been summarized by Nicola and Leach (1976a). From a study of solvent interactions with model tyrosine and tryptophan chromophores and thermal difference spectra of these models in various solvents, Nicola and Leach were able to classify the responses of chromophores in various protein environments into three main types. (1) Fully buried chromophores—those completely buried in the tightly packed, hydrophobic protein interior. Such residues were found not to contribute significantly to the protein difference spectrum presumably because of the low thermal expansivity of the protein interior. They were modeled by chromophores embedded in solid, optically transparent polyvinyl alcohol films which had been dehydrated (Nicola and Leach, 1976a). (2) Fully exposed chromophores—those completely hydrated and which can be modeled by the appropriate chromophore free in aqueous solution. (3) Partially exposed chromophores—such chromophores contribute to the protein difference spectrum but give rise to difference peaks at longer wavelengths than in 2; they can be modeled by the chromophore in any of a number of organic solvents or in mixed organic–aqueous solvents (Bello, 1969b; Smith, 1970; Leach and Smith, 1972; Nicola and Leach, 1976a).

To define the terms "fully exposed" and "partially exposed" and interpret our data on Mb[1] and Lb's it is necessary to have a model for the origin of the thermal difference spectra. The thermal difference spectra of tyrosine and tryptophan in aqueous solution are unique in that, in addition to spectral sharpening, they indicate that the parent spectrum is shifted to shorter wavelengths (blue shifted) upon cooling.[2] In any of the organic solvents, the parent spectra are either not shifted at all or shifted to slightly longer wavelengths (red shifted), a result more in keeping with theoretical predictions from the well-known "polarization red shift" arising from solute–solvent interactions. Laskowski (1969) has suggested that the anomaly may be related to the unique capability of water to form iceberg-like clusters around hydrophobic solutes, thus decreasing the density of water molecules around the chromophore as the temperature is lowered. Nicola and Leach (1976a), on the other hand, have presented evidence that the uniqueness of aqueous thermal difference spectra for tyrosine and tryptophan may result from the presence of specific hydrogen bonds where water acts as proton donor to the chromophore. With the former explanation, a chromophore would be recorded as fully exposed in a thermal difference spectral experiment only if a substantial fraction of the hydrophobic part of the chromophore were in contact with water, whereas with the latter explanation only the indolyl NH group of tryptophan or the OH group of tyrosine would need to be accessible to water. This can be an important distinction when interpreting protein difference spectra.

The difference spectra, which have been observed on cooling tyrosine and tryptophan models in various aqueous solvent ixtures (and in solid films), lead to the following generalizations.

When a leading difference peak is observed at higher wavelengths than that expected for a fully exposed chromophore, then that chromophore is probably surrounded by nonpolar groups which limit access to water but which are, nevertheless, motile (see, e.g., Figure 4a of Nicola and Leach, 1976a). The nonpolar groups are required for a shift to longer wavelengths and the motility is required to obtain a change in interaction strength of solute with its environment as the temperature is changed. The longer the wavelength of the difference peak, the more hydrophobic is the environment of the chromophore.

In those cases where the aromatic residues are either fully exposed or fully buried in the protein, the thermal perturbation method has given estimates of the fraction of each type which are in good agreement with known structures (Bello, 1969a,b, 1970; Smith, 1970; Leach and Smith, 1972); in more complicated situations involving partial exposure of some residues, it has also given estimates which agree with those obtained by other means (Nicola and Leach, 1976a). In this paper, the thermal perturbation method is applied to determine the exposure of aromatic chromophores to aqueous solvent in the holo- and apoproteins of sperm whale Mb and soybean Lb a. It is also applied for the first time to determine the accessibility of the heme group in the two proteins and to assess the contributions to the thermal difference spectra from changes in spin state of the iron.

Materials and Methods

Sources of proteins and chemicals, formation of apoproteins and liganded complexes, concentration determinations, and the binding of rose bengal to the apoproteins of Mb and Lb have all been described by Appleby et al. (1975), Nicola et al. (1975), and Nicola and Leach (1976b). Horse heart cytocrome *c* was a salt-free, highly purified material kindly given to us by Dr. R. W. Henderson.

Thermal Difference Spectra. A Cary 14 spectrophotometer was used for recording spectra; 0–1 A scale for parent spectra and 0–0.1 A scale for difference spectra with a dynode setting of 3, a scanning speed of 5–10 nm/min and a slit width of 0.1 or less. Optical densities were always less than 2 and the noise level usually less than ±0.003 A. Reference and sample cells (Hellma quartz QI, 1.000 cm) were independently thermostated in jacketed cell compartments, using two Colora thermostat baths, the reference cell at 25 °C, and the sample (double jacketed) at a variable temperature. For cooling the sample cell, a Colora refrigerated coil was inserted in a water–methanol bath and this was pumped through the sample-cell water bath. The time needed for temperature equilibration of the cells (30 min) was established by inserting thermistor probes directly into the solutions.

[1] Abbreviations used are: Hb, hemoglobin; Mb, myoglobin; Lb, leghemoglobin; UV, ultraviolet.

[2] Throughout this paper, reference is made to difference spectra indicating either a blue shift or a red shift. The former is a shift of the parent spectrum to lower wavelengths on cooling and is seen in the difference spectrum as a negative extremum on the long wavelength side of the parent absorption peak and a positive extremum on the short wavelength side. The latter is a shift of the parent peak to longer wavelengths and is seen in the difference spectrum as the mirror image of that observed for a blue shift. To both effects must be added the narrowing in bandwidth on cooling. The resultant difference spectra for the two cases are best visualized by constructing theoretical difference spectra or by using experimentally determined cooling difference spectra for model compounds (Nicola and Leach, 1976a).

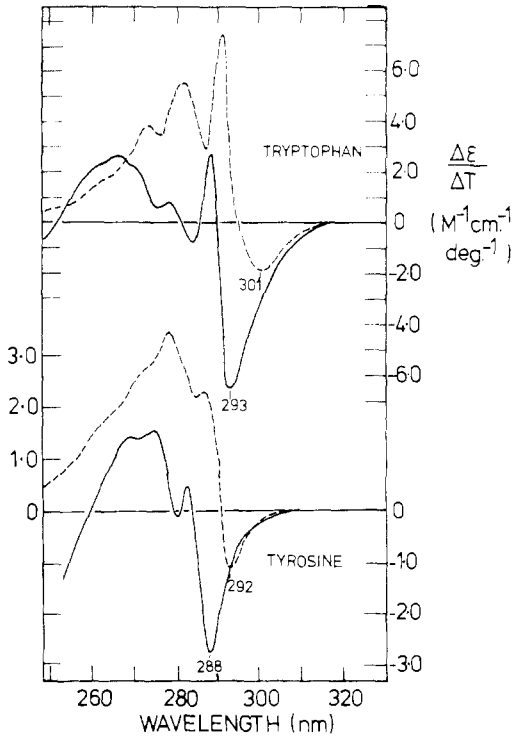

FIGURE 1: Thermal difference spectra of L-tryptophan (upper curve) and *N*-acetyltyrosine ethyl ester (lower curve); (—) in sodium phosphate buffer (0.1 M, pH 7.4) and (- - -) in 100% ethylene glycol. Difference spectra were calculated on a molar basis for a 1 °C temperature difference with sample temperature lower than that of the reference. All difference spectra were exactly linear with temperature difference over the range 5–40 °C.

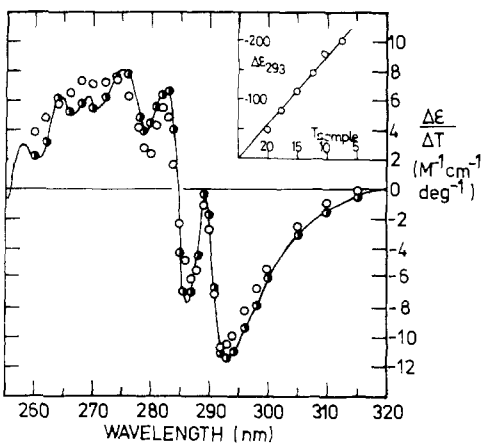

FIGURE 2: Thermal difference spectrum of soybean apoLb a in 6 M guanidine hydrochloride: (●–●) experimental curve; (○–○) calculated points for the summed contributions from two tryptophans and three tyrosines in the same solvent. Difference spectra were calculated on the same basis as those in Figure 1. Inset: dependence of the magnitude of the difference peak at 293 nm on the temperature difference between sample and reference cells. The reference cell was at 25 °C and apoLb a was 7×10^{-5} M.

Solutions were Millipore-filtered before running spectra. They were then left in the cell compartments for 30 min with both sample and reference solutions at 25 °C and a baseline was run. The temperature of the sample was then decreased (usually) in 5 °C steps and difference spectra were run at each temperature. The sample was finally returned to 25 °C in steps and difference spectra were again recorded at each step. A linear dependence of the amplitude of the difference spectrum on the temperature difference was taken as evidence that irreversible structural changes had not occurred during the experiment and that temperature equilibration was complete at each step.

Methods of Calculation. For the near-UV difference spectra the molar difference absorbance at certain wavelengths was plotted against the temperature difference between sample and reference. The slope of the line through these points $(\Delta\epsilon_\lambda/\Delta T)$ is the molar difference absorbance when the sample is 1 °C cooler than the reference. These quantities were calculated for the model chromophores in various solvents and for the proteins.

Since tyrosine gives little difference absorbance at 300 nm and tryptophan gives little difference absorbance at 288 nm in aqueous solution (Figure 1), these wavelengths were chosen to obtain approximate values of chromophore exposure in the proteins. Assuming that buried chromophores do not contribute to the difference spectra, one can write the simultaneous equations:

$$x(\Delta\epsilon_{300}/\Delta T)_{Trp} + y(\Delta\epsilon_{300}/\Delta T)_{Tyr} = (\Delta\epsilon_{300}/\Delta T)_{Protein} \quad (1)$$

$$x(\Delta\epsilon_{288}/\Delta T)_{Trp} + y(\Delta\epsilon_{288}/\Delta T)_{Tyr} = (\Delta\epsilon_{288}/\Delta T)_{Protein} \quad (2)$$

from which the number of exposed tryptophans (x) and tyrosines (y) can be calculated. The subscripts refer to the model for which the molar difference absorbance per degree was obtained. These numbers can then be refined and contributions from partially buried chromophores detected by curve fitting the experimental protein difference spectrum to the sum of the proposed model chromophore contributions (see, e.g., Figure 2). These computations were performed with an IBM 7044 computer.

For the thermal difference spectra recorded for the heme complexes in the Soret region, the main interest was in comparing such spectra from complex to complex and from one hemoprotein to another. Since the parent spectra of these complexes vary markedly with ligand (Nicola and Leach, 1976b), the difference spectra in these cases were normalized by dividing the molar difference absorbance per degree by the molar absorbance at the Soret peak $(\Delta\epsilon_\lambda/\epsilon_A)/\Delta T$.

Results and Discussion

The Near Ultraviolet. In most proteins there are four chromophores that can give rise to thermal difference spectra in the near-UV (250–320 nm), namely, tryptophan, tyrosine, phenylalanine, and cystine. In Mb and Lb's there are no cystines but there is the additional complication that the heme group absorbs in this region of the spectrum.

The thermal difference spectra of model tyrosine and tryptophan compounds in aqueous solution and in ethylene glycol are shown for reference in Figure 1. Tyrosine in aqueous solution is characterized by a leading negative difference peak at 288 nm and tryptophan by a negative peak at 293 nm. On a molar basis, the difference absorption due to tyrptophan is nearly three times that of tyrosine (note the scales in Figure 1) so that the difference spectra of Mb and Lb (two tryptophans and three tyrosines each) will probably be dominated by contributions from tryptophan. In an organic solvent, the leading thermal difference peak for both tyrosine and tryptophan decreases in intensity and is red shifted. At the same time, the positive difference absorbance at lower wavelengths (250–280 nm) becomes more intense. These difference spectra indicate a blue shift and band sharpening on cooling an aqueous solution of the chromophore but band sharpening and a slight red shift on cooling in organic solvents. Phenylalanine

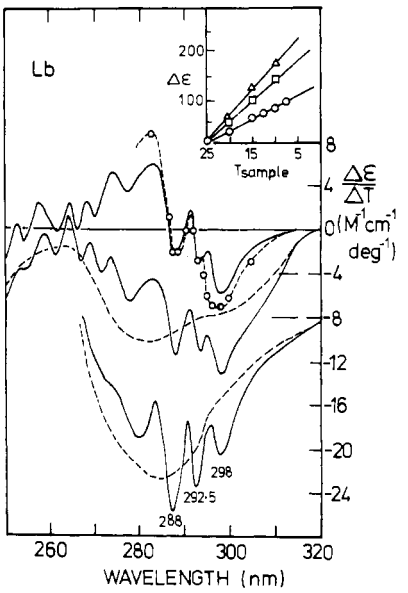

FIGURE 3: Thermal difference spectra of soybean Lb a complexes in the near-UV. From top to bottom, curves marked (—) are native apoLb a, fluoroferric Lb a, and cyanoferric Lb a; all are 7×10^{-5} M in potassium phosphate buffer (0.02 M, pH 7.2). The difference spectra for the fluoroferric and cyanoferric complexes have been offset vertically by −4 and −8 units, respectively, for clarity. (- - -) Difference spectrum due to heme obtained by subtracting out the difference spectrum due to apoLb. (O- -O) Calculated difference spectrum for the sum of three aqueous tyrosines, one aqueous tryptophan, and one tryptophan in 100% ethylene glycol. Inset: temperature dependences of the 298-nm difference peak for (O) apoLb a, (□) fluoroferric Lb, and (△) cyanoferric Lb. All difference spectra were calculated as in Figure 1. The reference cell was at 25 °C.

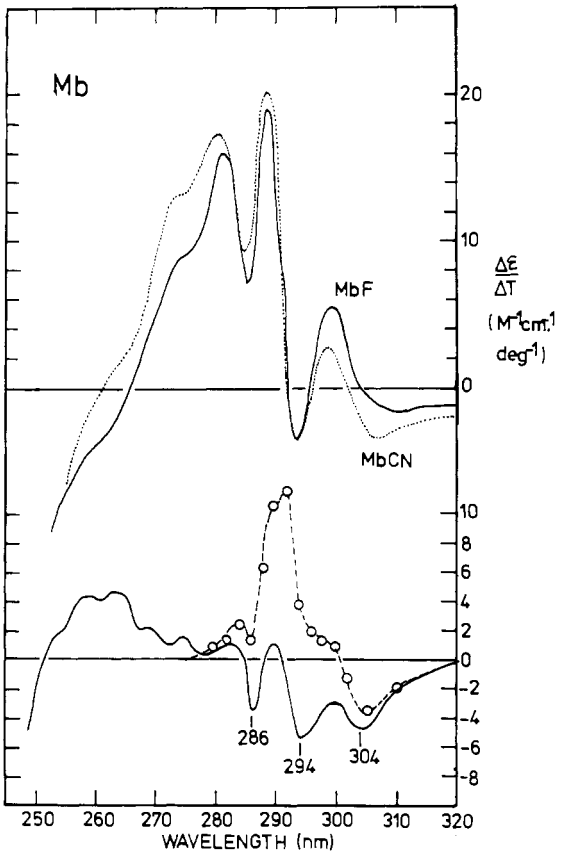

FIGURE 4: Thermal difference spectra of sperm whale Mb complexes in the near-UV. Upper curves: (—) fluoroferric Mb; (• • •) cyanoferric Mb. Lower curves: (—) apoMb. All are in potassium phosphate buffer (0.02 M, pH 7.2 for cyanoferric and fluoroferric; 0.1 M, pH 6.8 for apoMb). The curve marked O- -O is the difference between the curve for apoMb and that calculated for two aqueous tyrosines and one aqueous tryptophan. All difference spectra were calculated as in Figure 1.

gives the smallest difference peaks on a molar basis (not shown) and these occur at lower wavelengths (250–270 nm).

The simplest case to consider is that for the denatured apoproteins of Mb and Lb, since all the chromophores should be fully exposed to solvent and there is no heme absorbance. Figure 2 shows the experimental thermal difference spectrum for soybean apoLb a in 6 M guanidine hydrochloride and the difference spectrum obtained by summing the contributions from two tryptophans and three tyrosines in the same solvent. There is quite a good fit over the entire spectral range. The much smaller contribution from phenylalanine residues was ignored in this calculation but its presence can be seen as fine structure difference bands between 250 and 270 nm in the experimental difference spectrum. ApoMb in the same solvent (not shown) gave a very similar difference spectrum with $\Delta\epsilon/\Delta T = -5.4$ at 300 nm and −7.4 at 288 nm. Using eq 1 and 2, this gave 1.96 tryptophans and 3.8 tyrosines in this protein. Although the tyrosine value is somewhat high, this is in reasonable agreement with that expected, considering the much larger contribution from tryptophan.

Figure 3 shows the thermal difference spectra of soybean Lb a complexes, including the apoprotein, in the native state. A comparison of the difference spectrum for the native apoprotein with that for the denatured state (Figure 2) shows a significant difference—the long wavelength peak at 293 nm in the difference spectrum for the denatured protein is replaced with two peaks, one at 293 nm and one at 298 nm. Only tryptophan shows peaks as high as 298 nm and inspection of Figure 1 shows that this peak can be shifted up to wavelengths as high as 301 nm by a nonpolar, hydrogen-bonding solvent (ethylene glycol). The 298-nm difference peak is therefore assigned to a tryptophan whose indolyl NH group is not in contact with aqueous solvent but is in an environment of low polarity. The 292- and 288-nm difference peaks are assigned to tryptophan and to tyrosine residues, respectively, whose NH and OH groups are fully exposed to aqueous solvent. Using these assignments, a difference spectrum may be reconstructed as the sum of contributions from a tryptophan in aqueous solution, a tryptophan in ethylene glycol, and three tyrosines in aqueous solution. Figure 3 shows that such a difference spectrum does reproduce quite well the unusual features in the thermal difference spectrum of Lb. The calculated contribution near 300 nm is slightly greater in amplitude than the experimental but this can be decreased in model compounds by using a less nonpolar or stronger hydrogen-bonding solvent (Nicola and Leach, 1976a). The fine structure difference bands below 270 nm are again attributable to phenylalanines near the surface of the Lb molecule.

The thermal difference spectra for the high-spin fluoroferric and low-spin cyanoferric complexes of holoLb a (Figure 3) show that all the difference peaks seen for apoLb a are retained in the presence of the heme group, suggesting that the environments of the aromatic amino acids do not change significantly upon removal of the heme. By subtracting out the apoprotein difference spectrum from those for the holoproteins, one can see that the heme itself contributes negative difference absorption in the near-UV with the major peak near 285 nm.

A similar study of the thermal difference spectra of corresponding Mb complexes is shown in Figure 4. The thermal difference spectrum for the apoprotein is similar to that for

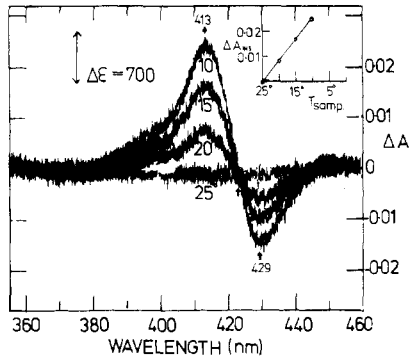

FIGURE 5: Thermal difference spectra of soybean cyanoferric Lb a (1.43×10^{-5} M) in potassium phosphate buffer (0.02 M, pH 7.05) in the Soret region. The reference cell was at 25 °C and the sample cell at the indicated temperatures. Each curve was scanned twice, once decreasing in temperature and once returning up again, to indicate the reproducibility and reversibility of the perturbation. Inset: temperature dependence of the magnitude of the difference peak at 413 nm.

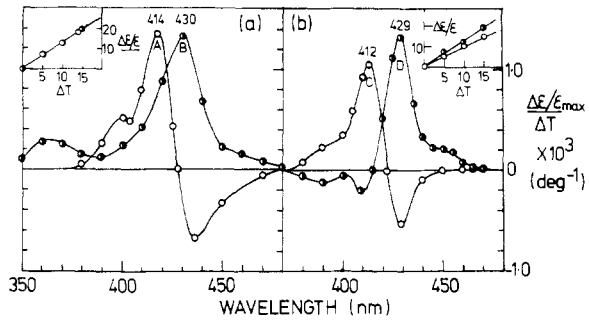

FIGURE 6: Normalized thermal difference spectra of: (a) cyanoferric heme (○) in water, (◑) and in 100% ethylene glycol; (b) (○) soybean cyanoferric Lb a, and (◑) sperm whale cyanoferric Mb, both in potassium phosphate buffer (0.02 M, pH 7.05). Proteins were $1.4-1.5 \times 10^{-5}$ M. Insets: temperature dependences of the major positive difference peaks.

apoLb, showing the same anomalous difference peak at high wavelengths, but for apoMb it is at an even higher wavelength, viz., 304 nm. The assignments for all these peaks are similar to those made for apoLb a, but the anomalous tryptophan is thought to be in an environment of even lower polarity than in Lb. This can be shown by subtracting out the contributions expected from one "aqueous tryptophan" and two "aqueous tyrosines" (the x-ray structure of sperm whale Mb has shown that one tyrosine is completely buried and the other two fully exposed (Kendrew, 1962)) from the experimental difference spectrum for apoMb. The resultant curve (Figure 4) shows a strong similarity to that for tryptophan in ethylene glycol (Figure 1), but is slightly larger and is shifted to longer wavelengths.

Subtraction of the apoMb difference spectrum from that for cyanoferric or fluoroferric holoMb does not give a smooth curve, but it can be seen by comparing the holo and apo spectra in Figure 4 that the heme group in Mb, in contrast to that in Lb, must be contributing positive difference absorbance to the difference spectrum, again centered near 280 nm for such a curve to be generated. Since ferriheme shows a major parent absorption band well below 280 nm (Urry, 1967), this indicates that the heme absorption in Mb is red shifted upon cooling, while that in Lb is blue shifted. This difference between Lb and Mb is also consistently observed for the heme bands in the Soret and visible regions and will be discussed in more detail later.

In summary, thermal perturbation difference spectra of the apoproteins and liganded holoproteins of soybean Lb a and sperm whale Mb in the near-ultraviolet reveal similarities in the tryptophan environments of the two proteins. Of the two tryptophans in each protein, one has its indolyl NH group fully exposed to aqueous solvent, while the other behaves as if it were surrounded by motile but nonpolar residues with little access to water. These environments are not significantly altered by the removal of the heme group, a conclusion reached also by Kirby and Steiner (1970) and Anderson et al. (1970) who studied tryptophan fluorescence in apoMb.

Inspection of the molecular model of sperm whale Mb (Kendrew, 1962) shows that the aromatic rings of both tryptophans are deeply buried. The NH group of Trp-A5 appears to be at the surface of the molecule with access to aqueous solvent. The NH group of Trp-A12 also appears to be near the surface, but it is surrounded by nonpolar side chains and access to water seems to be restricted. Trp-A12 occurs between the A and E helices and is nearly invariant in all the globins. It is thought to play an important role as a molecular spacer between these helices, helping to maintain the appropriate orientation of the E helix to the heme group. These environments agree well with those suggested from the thermal difference spectra. If, in solution, the tryptophans of Mb retain the environment seen in the x-ray structure, this would mean that only the indolyl NH of a tryptophan needs to be exposed to aqueous solvent for that residue to be recorded as fully exposed in a thermal difference experiment. This would support the origin of thermal difference spectra proposed by Nicola and Leach (1976a) rather than that of Laskowski (1969).

The two tryptophans of soybean Lb a (at positions 120 and 128) are at opposite ends of the amino acid sequence compared to those in Mb, yet they seem to have similar environments. The only difference is that the tryptophan in Lb, which shows very limited exposure to solvent, appears to be in a slightly less nonpolar and rigid environment to that for the corresponding tryptophan in Mb. There is the possibility of an interesting conformational homology between Mb and Lb, by which these two tryptophans may serve the same function in the two proteins. In soybean Lb a, as in *Chironomus* Hb III, Trp-A12 is replaced by a phenylalanine. Both proteins, however, have a tryptophan in position H8, while the other globins do not. Both positions H8 and A12 are internal sites and, although remote in linear sequence, could come very close to each other spatially in the protein interior.[3] The molecular model of sperm whale myoglobin (Kendrew, 1962) shows that a tryptophan at position H8 could serve the same function as one at position A12, namely, as the molecular spacer between the A and E helices. In *Chironomus* Hb, the x-ray structure shows that this may indeed be the case (Huber et al., 1971). The recent 5 Å resolution x-ray crystallographic study of lupin Lb (Vainshtein et al., 1975) shows very similar basic folding to that of the Mb's and Hb's, making the extension of the above generalizations to the Lb's quite reasonable. It therefore seems significant that the only Lb sequence examined that does not have a tryptophan at H8 (kidney bean; Lehtovaara and Ellfolk, 1975) is also the only one that does have a tryptophan at A12, while broad bean Lb (Richardson et al., 1975) has the same "switch" of the tryptophan to the H8 position as soybean Lb and *Chironomus* Hb.

The Soret and Visible Regions. In these regions of the

[3] Atanasov and Zhiznevskaya (1975) have also suggested that Trp-128 is the "exposed" tryptophan in lupin leghemoglobin, although Sievers and Ellfolk (1976) have shown that Trp-120 reacts more readily with *N*-bromosuccinimide.

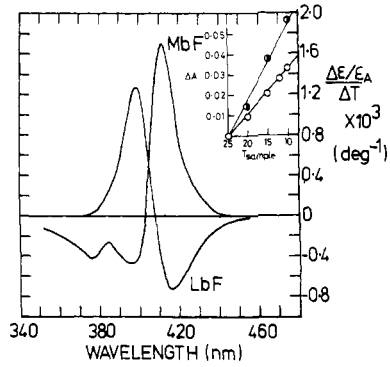

FIGURE 7: Normalized thermal difference spectra of sperm whale fluoroferric Mb and soybean fluoroferric Lb a in potassium phosphate buffer (0.02 M, pH 7.05, for Lb and 0.1 M, pH 6.8, for Mb). Protein concentrations were 1.2-1.5 × 10^{-5} M. Inset: temperature dependence of the major positive difference peak (◑) for MbF and (○) for LbF. The reference cell was at 25 °C.

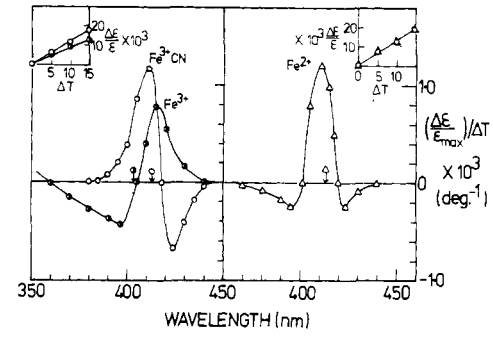

FIGURE 8: Normalized thermal difference spectra of horse heart cytochrome *c* derivatives: (◑) oxidized form; (○) cyanoferric form; (△) reduced form (with sodium dithionite). Symbols with arrows point to the wavelength of maximal absorption of the parent spectra. Insets: the dependence of the peak difference absorptions on the temperature difference between sample and reference cells (reference cell at 25 °C). For the inset on the right ○ refers to the temperature decreasing from 25 °C and △ refers to the temperature returning up again; all in sodium pyrophosphate buffer (0.1 M, pH 8.0) and protein concentrations were 8.7-15.7 μM. The reduction of cytochrome *c* was carried out under nitrogen.

spectrum, thermal difference spectra should reflect the environment of only the heme group. However, it is well known that some heme complexes (e.g., aquoferric, hydroxyferric, and azidoferric) are in a state of thermal equilibrium between low- and high-spin states, with lower temperatures favoring the low-spin state. Since the absorption spectra of low- and high-spin states are different (see, e.g., Nicola and Leach, 1976b), a change in temperature will give rise to a difference spectrum which, at least partially, reflects this transition (Beetlestone and George, 1964).

In studying the Soret and visible regions, the "spin effect" has been eliminated in several ways. First, thermal difference spectra of the cyanoferric and fluoroferric complexes of Mb and Lb, which are pure low-spin and pure high-spin, respectively, have been recorded. Second, they are compared to the difference spectra of ferrous, ferric, and cyanoferric complexes of cytochrome *c*, which are all low spin. Third, the heme group in Mb and Lb has been replaced with rose bengal (Nicola and Leach, 1976b) and this dye was used as a probe of the heme pocket, since it cannot show any changes in spin state. Finally, the contribution of thermal changes in spin state to the thermal difference spectrum has been evaluated for the aquoferric Lb a complex.

Figure 5 shows a typical thermal difference experiment for the cyanoferric complex of soybean Lb a in the Soret region. These difference spectra cannot be due to changes in spin state because (1) this complex is pure low spin, as measured by paramagnetic susceptibilities at all temperatures (Beetlestone and George, 1964; Ehrenberg and Ellfolk, 1963), and (2) the difference spectra indicate a shift of the parent spectrum to shorter wavelengths as the temperature is decreased, whereas a shift to longer wavelengths would be expected for a change to lower spin. They are also not due to dissociation of cyanide because of point 2 above and because identical difference spectra were obtained at several different cyanide concentrations (up to 100 times the molar concentration of hemoprotein). These difference spectra must therefore reflect the heme environment in the same way as do thermal difference spectra of tyrosine and tryptophan.

The results in Figure 6 indicate what the nature of the heme environment in Lb might be. It can be seen that the responses of the hemes in Mb and Lb to thermal perturbation are strikingly different. The difference spectra show that the parent sectrum of the heme in Mb is shifted to the red with decreasing temperature, while that for the heme in Lb is shifted to the blue. The difference spectrum for Lb is very similar to that of cyanoferric heme in water, while that for Mb is more like that of cyanoferric heme in ethylene glycol (in both of these solvents cyanoferric heme is monomeric). Figure 7 shows that this marked difference in response to thermal perturbation between Mb and Lb is also present in the high-spin fluoroferric complexes; i.e., the Mb spectrum is red shifted while the Lb spectrum is blue shifted upon cooling. The unique property of water to cause a blue shift in spectra upon cooling has also been noted for other chromophores (Pittz and Bello, 1970; Nicola and Leach, 1976a) and the result suggests that the heme in Lb has access to water molecules while that in Mb is in a hydrophobic environment.

Another model has been used to consolidate these assignments of heme environment in Mb and Lb. The x-ray structure of cytochrome *c* has shown that the heme group is inaccessible to water because both fifth and sixth coordination sites of the heme iron are occupied by protein ligands (histidine and methionine). Wüthrich (1971) has shown that added cyanide will replace methionine as sixth ligand and Schejter (1971) has shown that this reaction is entropically favored. The release of the methionine may cause an opening up of the heme crevice to allow ingress of aqueous solvent.

Figure 8 (left side) shows the thermal difference spectra of the ferric and cyanoferric derivatives of cytochrome *c*. These two difference spectra differ qualitatively in the way that those for Mb and Lb complexes differ (Figure 6b), indicating that the cyanoferric but not the ferric complex is accessible to water. Dickerson (1974) has suggested, from x-ray crystallographic studies, that reduction of cytochrome *c* leads to a tightening of the heme crevice with the complete exclusion of any water. The thermal difference spectrum for reduced cytochrome *c* (Figure 8, right side) does not look like any of the difference spectra discussed so far, but it indicates that, upon cooling, the parent spectrum is both sharpened and increased in intensity with little shift in the wavelength of peak absorption. This is the kind of effect expected when the only effect of decreasing the temperature is to reduce the conformational motility of the protein residues around the heme (see, e.g., Strickland et al., 1972).

To eliminate altogether any possibility of changes in spin or oxidation states or in bound ligand contributing to the thermal difference spectra of Mb and Lb, such difference

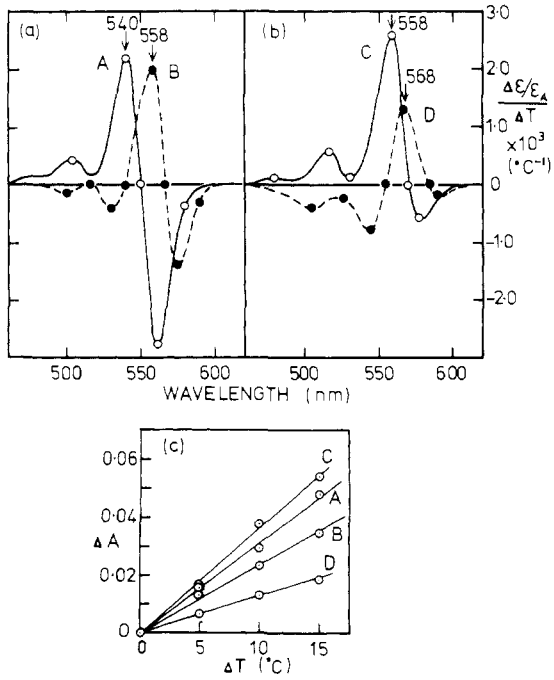

FIGURE 9: Normalized thermal difference spectra for rose bengal complexes. (a) Free rose bengal (—) in water and (- - -) in 100% ethanol. (b) Rose bengal bound to (—) soybean apoLb a and (- - -) sperm whale apoMb, both in potassium phosphate buffer (0.1 M, pH 7.0). In each case, rose bengal was 20 μM, apoLb a was 20 μM, and apoMb was 24 μM. (c) Temperature dependences of difference peaks labeled A, B, C, D. The reference cell was at 25 °C.

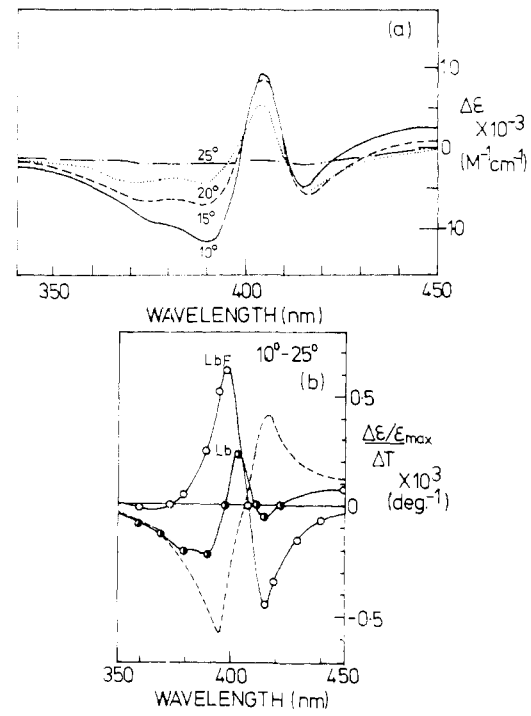

FIGURE 10: (a) Molar thermal difference spectra for soybean aquoferric Lb a in potassium phosphate buffer (0.02 M, pH 7.05). Protein concentration was 1.4×10^{-5} M and the reference cell was at 25 °C. Note that the temperature dependence of the difference spectrum is not linear. (b) (●) Thermal difference spectrum for aquoferric Lb a (taken from the 10–25 °C difference spectrum and normalized by dividing by the molar absorbance; (○) molar thermal difference spectrum for fluoroferric Lb a normalized as above; (- - -) the curve obtained by subtracting the fluoroferric difference spectrum from that for the aquoferric complex.

spectra have also been recorded for the complexes between rose bengal and apoproteins of Mb and Lb. Nicola and Leach (1976b) have adduced evidence that this dye binds in the vacant heme pocket of both proteins so that it can be used as a spectral probe of the environment in that pocket. Figure 9 shows the thermal difference spectra of the rose bengal complexes with apoMb and apoLb, and these are compared with those for the dye in water and in ethanol (a nonpolar solvent). The rose bengal–apoLb a complex again shows a difference spectrum qualitatively similar to that for the dye in water. The difference spectrum for the rose bengal–apoMb complex, on the other hand, is much more like that for the dye in ethanol. Since the major positive difference peak for the Lb complex is at lower wavelengths than the parent absorption peak, the difference spectrum for this complex indicates that a small blue shift occurs upon cooling (characteristic for aqueous solvents). However, the much smaller magntude of the long wavelength difference peak for the Lb complex compared to that for the dye in water shows that the heme pocket in Lb is not completely hydrated. The smaller magnitude of the Mb-complex difference spectrum compared to that for the dye in ethanol suggests that the heme pocket in this protein represents an environment more nonpolar and more rigid than that represented by ethanol.

Figure 10a shows the thermal difference spectra for the aquoferric complex of soybean Lb a in the Soret region. These are different from those reported for the other Lb complexes in two ways. First, they show a more complicated set of difference peaks and, second, the amplitudes of the difference peaks are not uniformly, linearly dependent on the temperature difference. This behavior is attributed to an additional effect besides that due to thermal changes in solute–solvent interactions and this is a thermal change in spin state. In Figure 10b, the thermal difference spectrum for fluoroferric Lb a, which has similar spectral properties to those for aquoferric Lb a so that it should represent the thermal perturbation component of the aquoferric difference spectrum, has been subtracted from the 10–25 °C thermal difference spectrum for aquoferric Lb a. The resultant curve is exactly that expected for a change to lower spin state upon cooling, indicating a red shift of the parent spectrum towards about 420 nm (the same wavelength at which the pure low-spin cyanoferric absorbs (Nicola and Leach, 1976b)). The molar difference absorption at this wavelength between aquoferric and cyanoferric complexes of Lb is about 45 000. The molar difference absorption per degree from the thermal difference spectrum calculated above for aquoferric Lb a, at the same wavelength, is $0.4 \times 150 = 60$ so that the thermal change in spin state is about 0.2% per degree. This is a reasonable value, since George et al. (1961) found, from paramagnetic susceptibility measurements, that hydroxyferric Mb, which is also known to be in a thermal equilibrium between high- and low-spin states, changes by 0.2% towards the low-spin state per degree of cooling over the temperature range 0–30 °C.

Figure 11 shows the thermal difference spectra of aquoferric soybean Lb a in the visible region. In contrast to those for the same complex in the Soret region, these difference spectra appear to be linear with temperature difference. The shape of these difference spectra is very similar to that obtained when a change from high to low spin in the ferric complex is produced by titration with nicotinic acid (Nicola and Leach, 1976b). However, if the thermal difference spectrum for aquoferric Lb a were due entirely to changes in spin state, the longest wavelength difference peak would be at 629 nm. The fact that it is at 640 nm (with a smaller component near 629

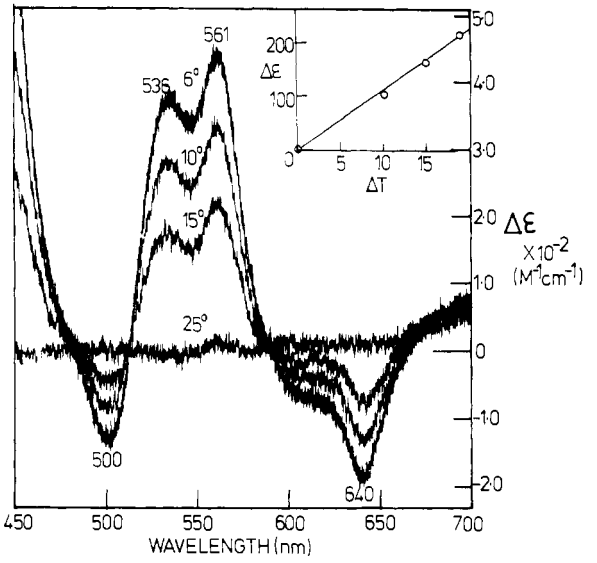

FIGURE 11: Thermal difference spectra in the visible region for soybean aquoferric Lb a in potassium phosphate buffer (0.02 M, pH 7.05). Lb was 1.21×10^{-4} M. The reference cell was at 25 °C and sample cell at the indicated temperatures. The difference spectra for 25–25 °C (baseline) and 6–25 °C were scanned twice. Inset: linear dependence of the difference peak at 561 nm on temperature difference.

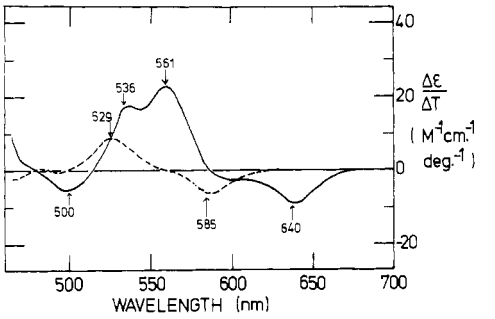

FIGURE 12: Molar thermal difference spectra of (—) aquoferric and (- - -) cyanoferric soybean Lb a derivatives in the visible region. Both were 1.2×10^{-4} M in potassium phosphate buffer (0.02 M, pH 7.5) and showed a linear temperature dependence.

nm) suggests that the parent spectrum is blue shifted upon cooling (the thermal perturbation effect), indicating an aqueous heme environment. The linearity of the difference spectra in Figure 11 can then be explained because both the perturbation (difference peak at 640 nm) and the spin-state effects (difference peak at 629 nm) are linear with temperature difference and reinforce each other, whereas in the Soret region the two effects have a canceling effect.

That a blue-shift component is present in the visible thermal difference spectra for the aquoferric complex is indicated in Figure 12. The pure low-spin cyanoferric complex of soybean Lb a shows just the kind of difference spectrum expected for a blue shift of the parent spectrum upon cooling.

To summarize, thermal difference spectra in the Soret and visible regions of the spectrum have given information on the differences in heme environments between Mb and Lb. The blue shift represented by the cooling thermal difference spectra of Lb complexes reflects an aqueous environment around the heme group, while the red shift observed for Mb complexes reflects a predominantly nonpolar environment around the heme. It is uncertain how many water molecules would have to penetrate the heme pocket to cause the heme to behave in the same way as it does when free in aqueous solution. This would depend on whether the diagnostic blue shift observed upon cooling an aqueous solution of heme is caused by a bulk solvent effect or by some specific interactions between the heme and one or two water molecules (as is probably the case for tyrosine and tryptophan). Certainly, the heme pocket in Lb cannot be completely hydrated, since the iron in free heme in aqueous solution is autoxidized too rapidly to bind oxygen. The results with rose bengal in Lb support this conclusion.

Nevertheless, it does seem very likely that the heme pocket for all the Lb complexes contains some water molecules, whereas that in Mb contains fewer or none at all. This is an important result because recent studies with model heme complexes (Brinigar et al., 1974; Stynes and Ibers, 1972; Chang and Traylor, 1975) have shown that, contrary to expectations, a more polar (aqueous) environment around the heme increases the oxygen affinity of the heme. This could be one important reason for the much higher oxygen affinity of Lb's compared to Mb and Hb's.

It is difficult to say whether there is any difference in the heme environment between the low- and high-spin complexes of Lb and Mb. The thermal difference spectra for the high-spin complexes of both Lb and Mb are slightly larger in magnitude than those for the low-spin complexes, even after normalization. This may reflect a more flexible heme pocket in the high-spin state, as suggested by circular dichroic results (Nicola et al., 1975).

Taking these results in conjunction with those from circular dichroism (Nicola et al., 1975) and absorption spectroscopy (Nicola and Leach, 1976b), one may postulate a plausible structural basis for the higher oxygen affinity of Lb's compared to Mb.

The more polar heme cavity in Lb will stabilize the iron–oxygen bond which is thought to be dipolar and will promote deprotonation of the δ1-NH group of the proximal histidine, making it more basic. On the basis of model compound studies (see, e.g., Chang and Traylor, 1975) this should increase the oxygen affinity of Lb relative to Mb. The more open and flexible character of the heme pocket in Lb should provide less steric hindrance to oxygen entry and, perhaps more importantly, permit the high- to low-spin transition required for oxygenation to occur more readily.

It is possible that the differences in heme environments between Lb and Mb are attributable to the deletion of Tyr H22[4] and functional substitution of Trp A12, possibly by Trp H8 in most Lb's. The deletion of Tyr H22 in Lb's would both increase the solvent accessibility to the heme and probably decrease the free energy of binding oxygen, since oxygenation of Hb chains (Perutz, 1970) and probably of Mb results in the rupturing of a hydrogen bond that this tyrosine makes in the deoxy state and a rotation of this tyrosine from a buried to a solvent-exposed situation. Pursuing the conformational homology indicated by the x-ray data of Vainshtein et al. (1975): the functional replacement of Trp A12 between helices A and E may well weaken the strength of interaction between the E helix and the heme group which is probably the main determinant of flexibility in the heme pocket. This would both increase the accessibility of the heme group to solvent and decrease the free energy of the high- to low-spin transition required for the oxygenation process (Perutz, 1970).

Acknowledgments

The authors are grateful to Dr. C. A. Appleby (Division of Plant Industry, CSIRO, Canberra) for his guidance in the

[4] The nomenclature used for residue positions and helices is that of Watson and Kendrew (1961) for sperm-whale Mb.

preparation of soybean Lb a and its derivatives, and Dr. R. W. Henderson for a gift of purified horse heart cytochrome *c*.

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