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TEMPERATURE DEPENDENCE IN THE ABSORPTION SPECTRA OF BEEF LIVER CATALASE

William R. BROWETT * and Martin J. STILLMAN *,**

Department of Chemistry, University of Western Ontario, London, Ontario N6A 5B7, Canada

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The spin characteristics of the ferric heme groups in native beef liver catalase, and in the complexes formed by reaction with fluoride, cyanide, azide, thiocyanate, and cyanate ions have been studied using absorption spectroscopy over the temperature range of 4–285 K. The azide, isothiocyanate, and isocyanate complexes of catalase are considered to be high-spin ferric heme complexes at room temperature, but undergo a thermal spin change below 300 K. The temperature dependence of these absorption spectra, however, cannot be analyzed in terms of simple Boltzmann distributions between two $S = 1/2$ and $S = 5/2$ spin states. The data show that these spin changes occur over a very narrow temperature range, but do not result in the formation of completely, low-spin complexes. The data also suggest that the thermal spin changes that occur below the glassing temperature of the solvent are dependent upon the conformational changes which take place within the protein itself with a change in temperature, and which directly affect the environment of the heme group.

1. Introduction

Unlike hemoglobin, myoglobin, or horseradish peroxidase in which a proximal histidine amino acid is axially coordinated to the heme iron, the tetrameric heme enzyme catalase (EC 1.11.1.6), which decomposes hydrogen peroxide according to:

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad [1]$$

contains a phenolate amino acid coordinated to the predominantly high-spin ferric heme [2]. However, like many ferric heme-proteins, catalase will form complexes with intermediate field ligands such as azide, and in these complexes there will be a thermal equilibrium between a ferric high-spin ($S = 5/2$) and a ferric low-spin ($S = 1/2$) species [3–6]. The spin states of the iron in ferric heme-proteins and porphyrins have been correlated with the structure of the ligands around the iron where, if d represents either the distance of the iron out of the heme plane domed towards the covalently bound amino acid [7,8], or the porphyrin core size [9], then

$$d_{\text{low-spin}} < d_{\text{intermediate-spin}} < d_{\text{high-spin}}.$$

A thermal spin equilibrium will result in a mixture of configurations which will be joined by conversion through the many conformers found for the conformationally dynamic heme proteins [10]. Thus, a spin equilibrium can be associated with a change in conformation of both the heme and the adjoining amino acids through the axially coordinated residue. The magnetic and spectral properties of this species will be intermediate between the purely high- and low-spin species, and distinct from the true intermediate-spin species which has a single spin of $S = 3/2$, or the quan-

* Associated with the Centre for Interdisciplinary Studies in Chemical Physics at the University of Western Ontario.
** To whom correspondence should be addressed.

tum-mechanical mixed-spin $S = 5/2$ and $3/2$ species. The latter has been proposed for the electronic ground state of the heme in native cytochrome c' and in native horseradish peroxidase [7,11,12].

In this paper, we describe the analysis of absorption spectra recorded for complexes of catalase with azide, thiocyanate, and cyanate ligands. In each case the absorption spectra show that a mixture of spin states is present over the temperature range 4–285 K. We observe in the spectra of these catalase complexes behavior similar to that of crystalline iron complexes in which spin conversion hystereses may be manifest by incomplete spin conversion or a conversion over a very narrow temperature range. These observations may be contrasted with the simple, linear $\ln K$ vs. $1/T$ relationship observed for the equilibrium between the $S = 1/2$ and $S = 5/2$ spin states in most heme-proteins [13].

The compensation temperature, T_c, which is defined as the midpoint of the thermal spin conversion, has been estimated for each of the catalase species, and compared to values obtained for other heme-proteins. The compensation temperature values for catalase complexed with intermediate field ligands are at or below the solvent glassing temperature of 220 K, whereas the comparable values observed for myoglobin, cytochrome c peroxidase, or horseradish peroxidase complexes are characteristically between 230 and 400 K [14,15]. The catalase absorption data measured for this study correlate well with our earlier room temperature magnetic circular dichroism (MCD) results for a variety of ligands bound to ferric catalase where a trend towards a high-spin ferric heme was identified [16]. The MCD spectra were found to be a sensitive indicator of the fraction of low- and high-spin species present at room temperature.

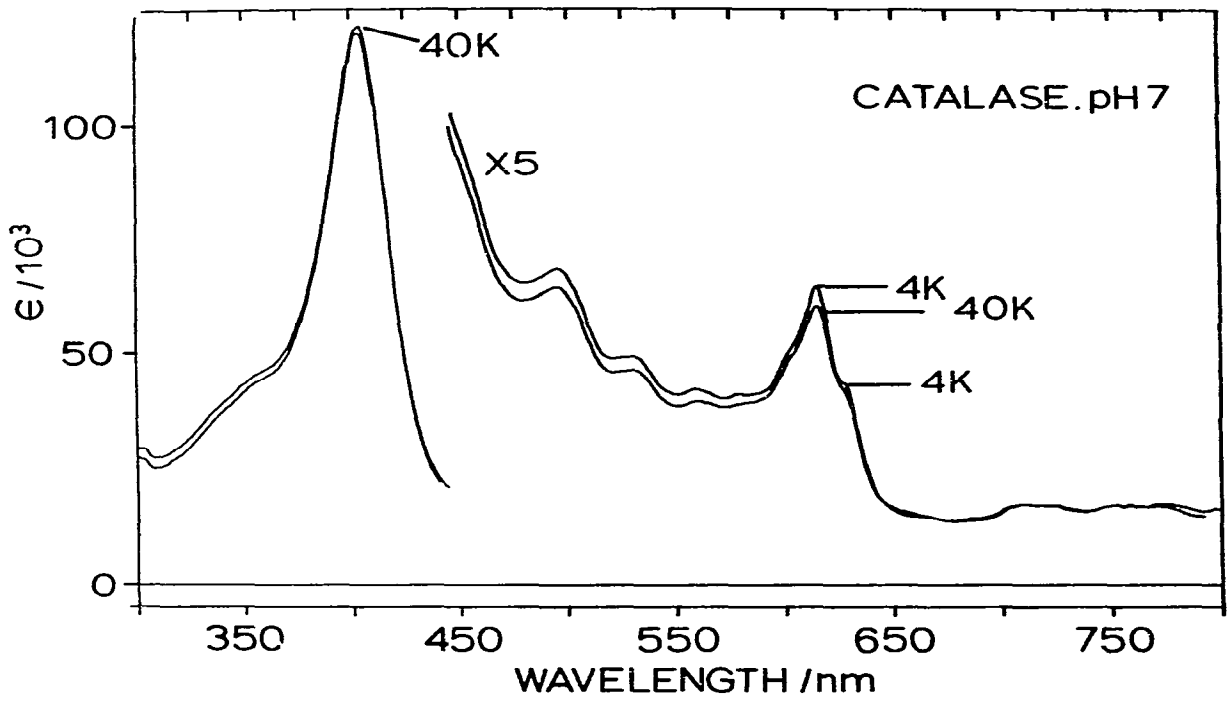

Fig. 1. The low-temperature absorption spectra of catalase in a phosphate buffer at pH 7, $I = 0.05$, dissolved in glycerol to form a 1 : 1 (v/v) solution. At room temperature $A_{405} = 1.16$.

2. Experimental

Stock solutions of beef liver catalase (Boehringer Mannheim, received as a crystalline suspension in 0.01% alkyl benzyldimethylammonium chloride), were prepared by exhaustive dialysis against the final buffer, either phosphate (pH 7, $I = 0.05$) or lactate (pH 5, 0.02 M) buffers. Aliquots of these solutions were added by mass to obtain 1 : 1 (v/v) H_2)/glycerol solutions. The solutions were thoroughly mixed, and centrifuged to remove the bubbles which would interfere with the glassing process. Complexes were prepared by dissolving the appropriate weight of salt in a catalase/glycerol solution.

The low-temperature absorption spectra were obtained using a Cary 219 spectrophotometer, and an Oxford Instruments CF204 (Oxford, U.K.) exchange gas optical cryostat. The solutions were placed in 1 cm acrylic cuvettes (Fisher Scientific Co.). A baseline of the absorption characteristics of a glassed H_2O/glycerol solution in an acrylic cuvette and the cryostat at 80 K was digitally subtracted from each spectrum of the glassy samples. All spectra were automatically digitized as described previously [16] only after the temperature had stabilized and no further spectral changes were observed. The volume changes that occur as the solutions cooled have not been taken into account in reporting the absorbances in the spectra shown here. The positioning of the baselines in the isocyanate and isothiocyanate spectra required the use of isosbestic points at 600 and 595 nm (isocyanate) and 340 nm (isothiocyanate).

3. Results

The low-temperature absorption spectrum of native ferric catalase at pH 7, and the spectra of

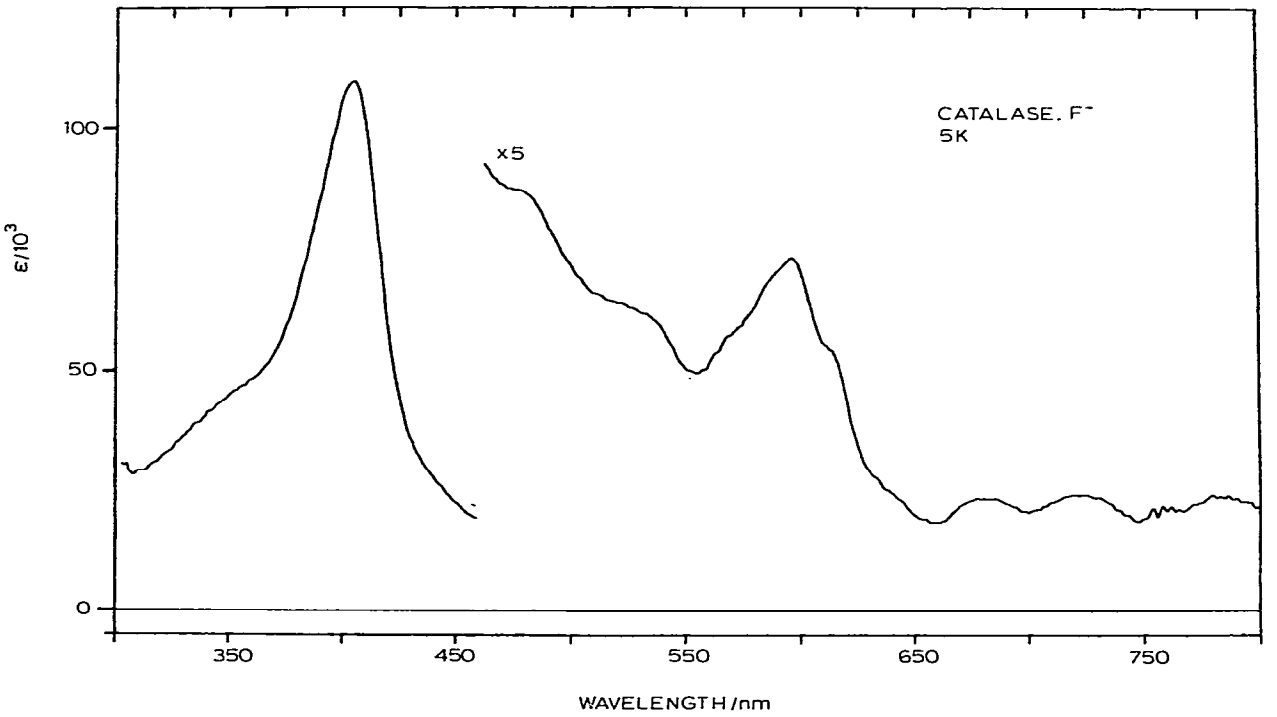

Fig. 2. The 5 K absorption spectrum of a glassed 1 : 1 (v/v) catalase fluoride/glycerol solution; [NaF] = 0.5 mol l^{-1}. This spectrum was identical to the 100 K spectrum. At room temperature $A_{405} = 1.11$.

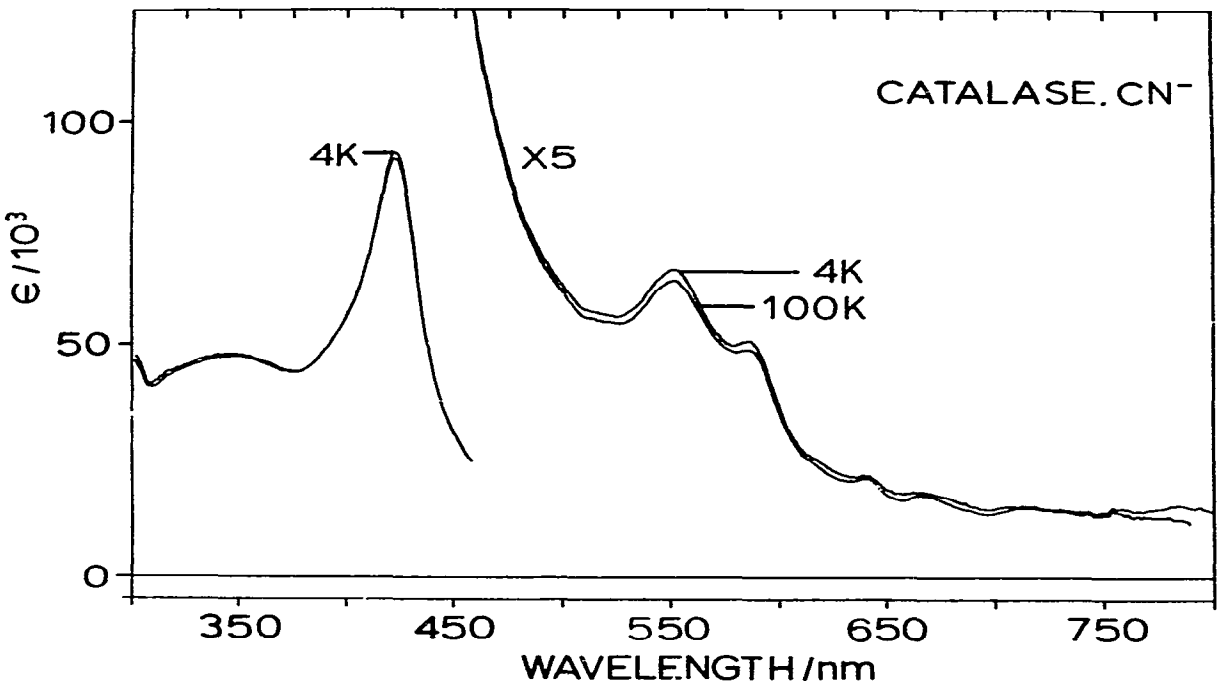

Fig. 3. The low-temperature absorption spectra of glassed 1:1 (v/v) catalase-cyanide/glycerol solution; [NaCN] = 0.01 mol l^{-1}. At room temperature $A_{423} = 1.08$.

complexes of catalase with fluoride, cyanide, azide, isothiocyanate, and isocyanate ligands in aqueous/glycerol solutions are shown in figs. 1–6. The spectrum of native catalase at pH 7, shown in fig. 1, is only slightly temperature dependent with a general shift to higher energy as the bandwidths narrow at the lower temperatures. At 40 K, the blue shift of the 622 nm room-temperature band to 614 nm is accompanied by an increase in resolution that reveals the multi-transition nature of this region of the spectrum. An increase in resolution and intensity is also observed in the charge-transfer band at 600 nm in the spectrum of catalase fluoride between 295 and 100 K; the spectrum at 5 K is identical to that at 100 K, shown in fig. 2. Similarly, the spectra of the cyanide complex recorded at 100 and 4 K are also almost identical, both show a considerably sharpened Q band near 550 nm, shown in fig. 3. At 4 K, the Q_{00} band (fig. 3) clearly appears as a shoulder at 585 nm on the 551 nm Q_{vib} band which has shifted to 551 nm from 554 nm at room temperature, while the B band position remains unchanged at 423 nm.

The most complicated temperature-dependent changes occur in the absorption spectra of the catalase-azide, -isothiocyanate and -isocyanate species. (It is assumed, that, like the thiocyanate ligand of the myoglobin-isothiocyanate complex, the cyanate and thiocyanate ligands bind to the heme iron through the nitrogen atom [17].) A thermal equilibrium between high- and low-spin configurations clearly exists in these complexes. The predominantly high-spin species at room temperature convert to heme configurations which are predominantly low-spin below 50 K for catalase-azide, and below 170 K for the isothiocyanate species (figs. 4 and 5, respectively). In contrast, the conversion of the catalase isocyanate species (fig. 6), to a low-spin species is far less complete, even by 10 K.

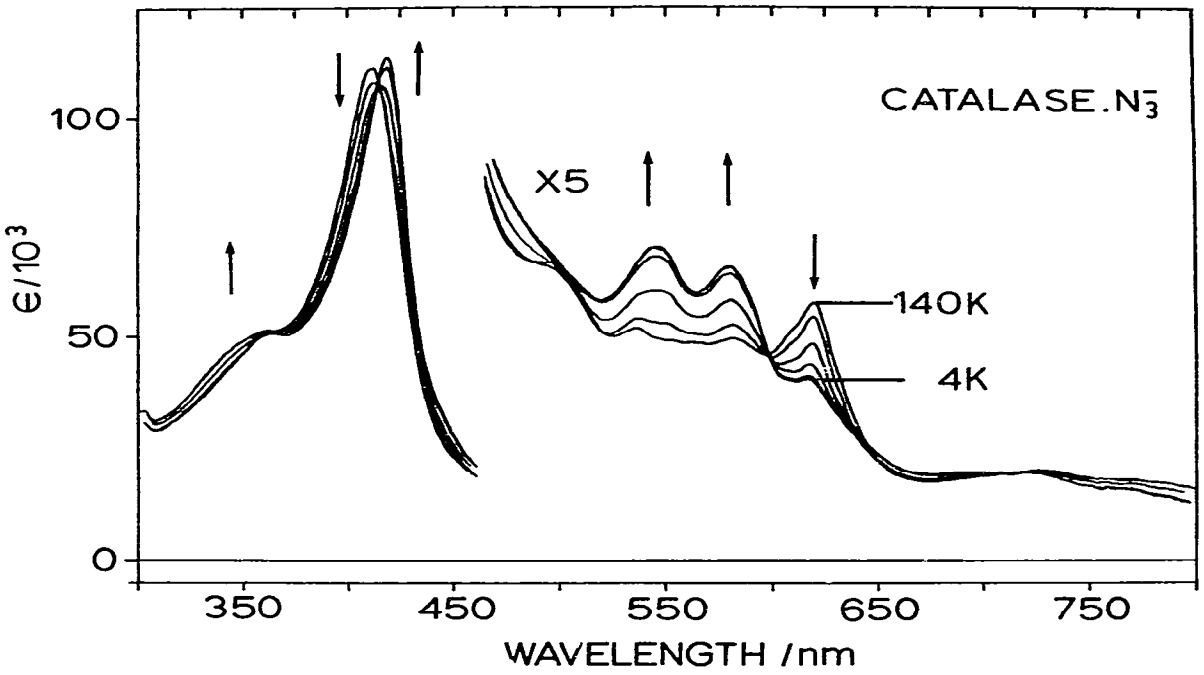

Fig. 4. The low-temperature absorption spectra of a glassed 1 : 1 (v/v) catalase-azide/glycerol solution; $[NaN_3] = 0.05$ mol l^{-1}. At room temperature $A_{411} = 2.35$.

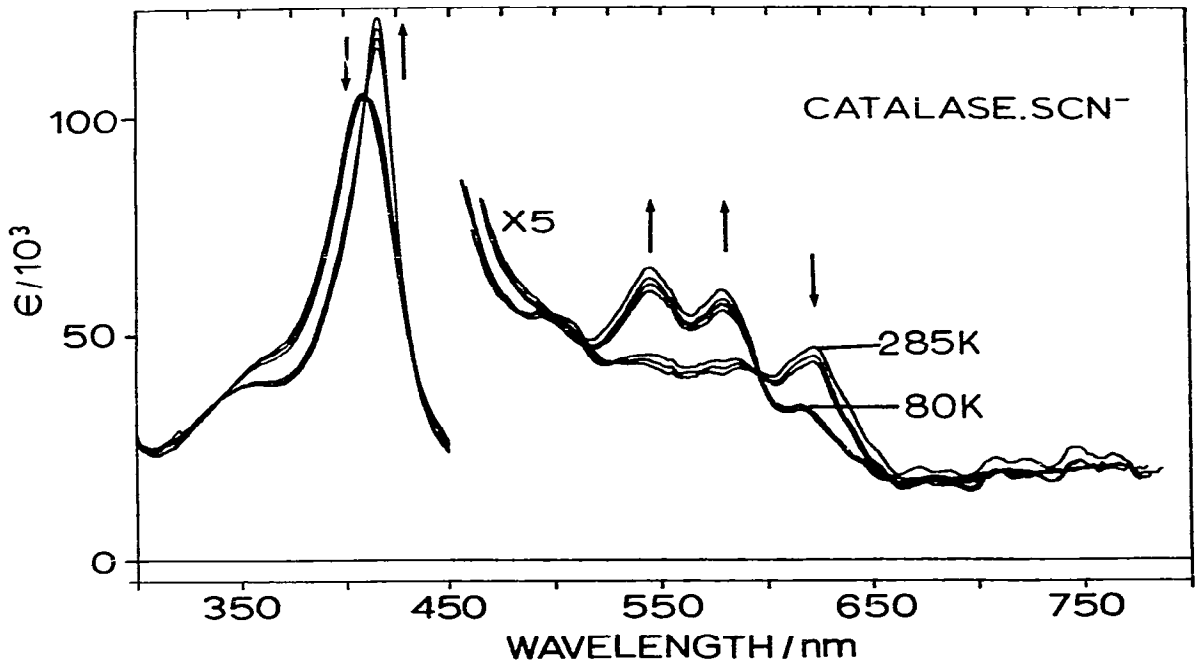

Fig. 5. The low-temperature absorption spectra of a 1 : 1 (v/v) catalase-isothiocyanate/glycerol solution; [NaCNS] = 0.05 mol l^{-1}. At room temperature $A_{408} = 1.08$.

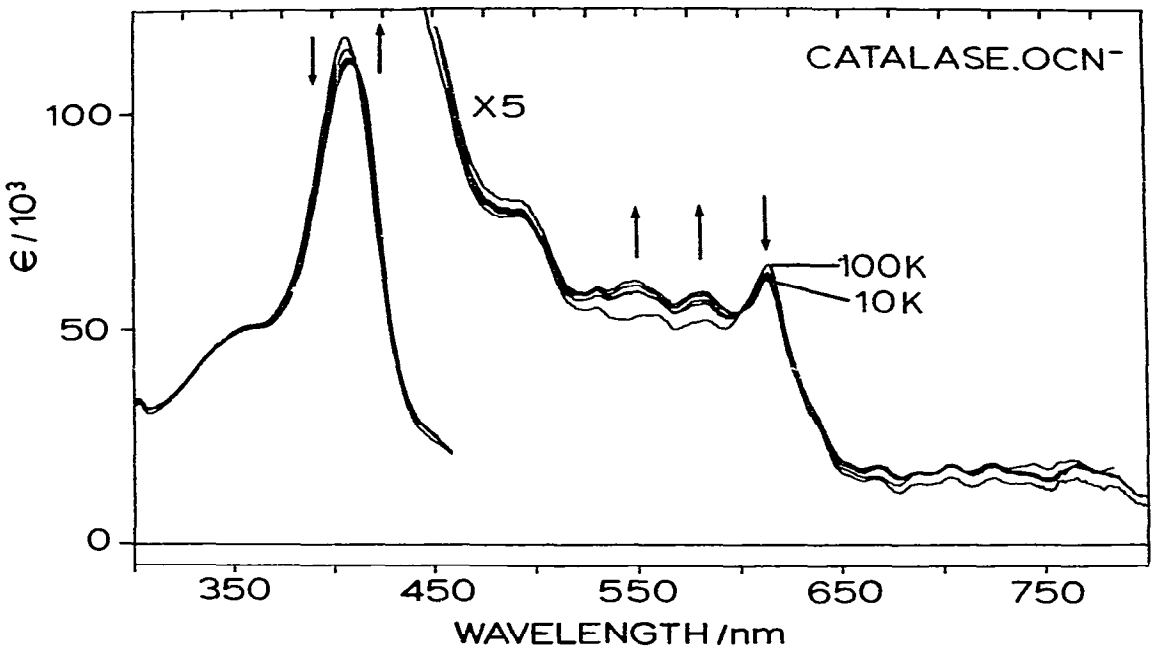

Fig. 6. The low-temperature absorption spectra of a glassed 1 : 1 (v/v) catalase-isocyanate/glycerol solution; [KCNO] = 0.05 mol l^{-1}. At room temperature $A_{406} = 1.33$.

The high-spin spectrum for catalase-azide (measured at 140 K) has a B band at 411 nm, with charge-transfer bands at 494 and 616 nm, of which the latter is shifted from the room-temperature position of 620 nm. The high-spin species is in thermal equilibrium with a low-spin species which has a B band at 419 nm, Q_{vib} at 545 nm, Q_{00} at 579 nm and a shoulder at 616 nm. The 616 nm band is associated with the high-spin component of this complex. The spectrum of the high-spin

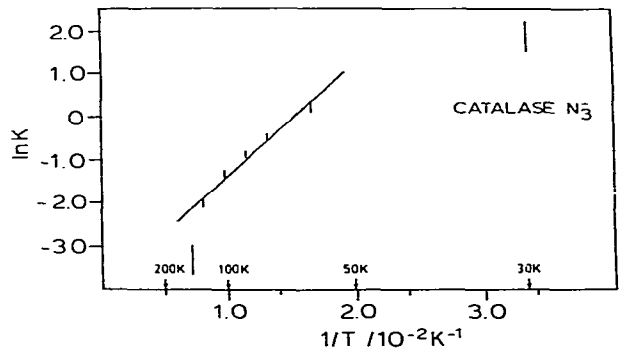

Fig. 7. A plot of ln K vs. $1/T$ for the catalase-azide complex. An equilibrium constant, K, was determined at each temperature for the bands at 547, 579 and 617 nm.

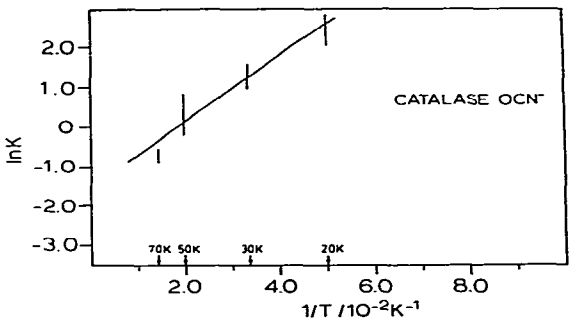

Fig. 8. A plot of ln K vs. $1/T$ for the catalase-isothiocyanate complex. An equilibrium constant, K, was determined at each temperature for the bands at 545 and 579 nm.

species of the catalase-isothiocyanate complex at 285 K (fig. 5) has distinct absorption bands at 409, 494 and 621 nm, while the low-spin species is characterized at 10 and 80 K by a spectrum with a B band at 416 nm, Q_{vib} at 544 nm, Q_{00} at 578 nm and a low-energy shoulder at 618 nm. The catalase-isocyanate complex (fig. 6), which at 100 K has a B band at 407 nm, and charge-transfer bands at 493 and 614 nm, is still a predominantly high-spin complex. The temperature-dependent changes in the spectra of the isocyanate complex are significantly smaller than the changes observed in the azide and isothiocyanate complex spectra, and are characterized by a shift of the B band to 409 nm from 407 nm, and a decrease in intensity at both 490 and 614 nm, together with a corresponding increase in intensity of the Q bands as the temperature is reduced from 100 to 10 K.

The thermal spin equilibrium can be described in terms of an equilibrium constant, K, where

$$K = (A - A_{HS})/(A_{LS} - A)$$

and A_{HS} and A_{LS} are the extremes of the high- and low-spin absorption intensity of each species, respectively, at a specific wavelength. The changes in the equilibrium constant, ln K, with inverse temperature for the catalase-azide, -isothiocyanate and -isocyanate complexes are illustrated in figs. 7, 8 and 9, respectively. For these species there is no simple Boltzmann relationship between the equilibrium constant and the temperature which would be indicated by a linear relationship between ln K and $1/T$. The region of temperatures in which the high- and low-spin species of the catalase-isothiocyanate complex are in approximately equal concentrations could not be studied, as this occurs near the glass transition point of the solvent at about 190–210 K. Although the spectral changes associated with the thermal spin conversion of the catalase-isocyanate complex are qualitatively distinct, the quantitative determination of the equilibrium constant was made difficult by the experimental conditions and the small magnitude of the spectral changes, as indicated by the large variation in the equilibrium constant determined for several wavelengths in the spectrum at each temperature.

The values of T_c, the temperature for the mid-

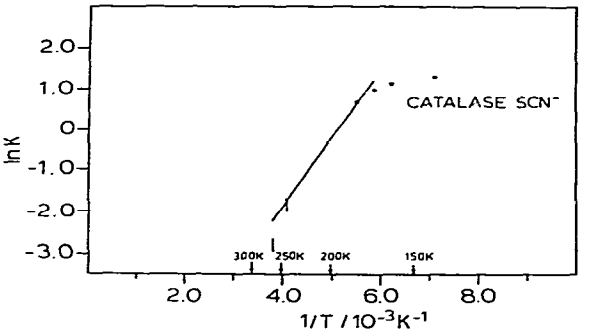

Fig. 9. A plot of ln K vs. $1/T$ for the catalase-isocyanate complex. An equilibrium constant, K, was determined at each temperature for the bands at 550, 580 and 614 nm.

point of the thermal spin conversion, were estimated using the value of $K = 1$ and ln $K = 0$ from the plots in figs. 7–9, for the equilibrium constant. The compensation temperatures have been calculated for the catalase-azide, -isothiocyanate, and -isocyanate species, and are listed in table 1. Overestimation of the absorption intensity associated with the spectra recorded at the lower temperatures may occur due to solvent contraction and vibronic or band narrowing effects (as observed in the spectra of the spin-stable catalase complexes) will skew the T_c values to higher temperatures.

Table 1

The compensation temperature values, T_c, for several heme protein complexes, and estimated values for the catalase-azide, -isothiocyanate and -isocyanate complexes

	T_c(K)	Reference
Myoglobin N_3^-		
horse	375	21
sperm whale	383	21
Hemoglobin N_3^-		
human	400	21
Cytochrome *c* peroxidase N_3^-	298	15
Horseradish peroxidase N_3^-	327	14
Catalase		
horse erythrocyte N_3^-	87	33
beef liver N_3^-	64	this work
beef liver NCS^-	190	this work
beef liver NCO^-	51	this work

4. Discussion

The low-temperature absorption spectra described above are in essential agreement with the spectra obtained between room temperature and 77 K for the cyanide, azide, fluoride and native pH 7 horse erythrocyte catalase species [3–5]. No further significant change is observed in the cyanide, fluoride or native pH 7 catalase spectra as the temperature is lowered to 10 K. However, at 10 K the spectra of the beef liver catalase-azide and -isothiocyanate species are predominantly low-spin in appearance, and can be readily distinguished from the spectrum of the completely low-spin cyanide complex by the clear resolution of the $Q_{(0)}$ band from the $Q_{v(b}$ band. The well resolved $Q_{(0)}$ band also appears to be a feature of the spectrum of horse erythrocyte catalase at pH 11 [3], and the ammonia complex of bacterial catalase [6,18]. The bacterial catalase ammonia, methylammonia, and the ethylammonia complexes [18] undergo a thermal spin change to a low-spin species by 220 K. The methyl- and ethylamine complexes apparently have a greater fraction of the low-spin species present than do the azide, isothiocyanate, or isocyanate species at room temperature as indicated by the lack of intensity in the 620 nm high-spin marker band.

In all spectra of the spin-labile species of catalase there is residual absorption in the 620 nm region of the spectrum that may be associated with the 620 nm charge-transfer transition of the high-spin species. However, the spectral characteristics of the predominantly low-spin complexes of each of these species, and the purely low-spin catalase-cyanide complex, are very similar to those obtained for the iron tetraphenylporphyrin–2-methylimidazole complex in which the iron–imidazole nitrogen bond strain is associated with intensity observed in the 630 nm region of the spectrum; the region of the spectrum for which intensity is normally associated with absorbance from the charge-transfer band of a high-spin iron heme complex [19].

Heme-proteins are notable for having both high- and low-spin ground states that may be related through a thermal spin conversion. The position of the equilibrium depends on both the axial ligand on the ferric iron and on the protein environment of the heme. For example, the myoglobin-isocyanate complex has a high-spin ground state whereas the cytochrome *c* peroxidase-isocyanate complex has low-spin ground state. For those heme-proteins which have been studied, a range of T_c values have been assembled according to the parent protein [8]. These T_c values range from 331 to 388 K for myoglobin (with the exception of the myoglobin-isocyanate complex which has $T_c = 255$ K [13]), from 372 to 415 K for hemoglobin [20,21], from 232 to 298 K for cytochrome *c* peroxidase [15,22], and from 247 to 327 K for horseradish peroxidase [14]. The T_c values fo. the catalase complexes that have been estimated using the maximum and minimum absorption values obtained from the experimental data are clearly more variable and lower than the literature T_c values for other heme proteins. The literature T_c values were calculated from absorption, or magnetic susceptibility data by assuming limiting absorption or susceptibility values for complete conversion to high-spin and low-spin complexes.

The ligand-field description of the spin conversion in octahedral iron complexes predicts that the transition from a high-spin to a low-spin configuration will depend on whether the ligand-field splitting is greater or less than the interelectron repulsion energy. A simple, thermal spin-transition model would associate a linear dependence of ln K with an inverse temperature for the equilibrium between the $S = 1/2$ and $S = 5/2$ spin states of the iron(III) that are separated by an energy of approximately kT. In addition, the spin conversion process involves a conformational change of the porphyrin which can best be described in terms of a radiationless, multiphonon process occurring between two spin states which are characterized by different equilibrium configurations [23].

There have been several analyses of spin conversions in both ferric and ferrous iron complexes which have shown that the average reduction in bond length of the inner coordination sphere is 12 pm [24–29]. The bond length reductions which have been observed in iron porphyrin complexes [30] have also been associated with a change in electronic occupation of the $3d_{x^2-y^2}$ orbital [30,31]. Thus, a trend in iron–nitrogen bond lengths of

206.8(8), 200.1(5) and 199.0(1) pm has been observed for a series of high-, intermediate- and low-spin porphyrin complexes, respectively [31]. Although the doming of the iron out of the plane of the ring occurs in five-coordinate, high-spin iron-porphyrin complexes, coordination by two weak field ligands, such as a neutral oxygen group in a ligand, may result in an in-plane stereochemistry with substantial radial expansion of the porphinato core relative to low-spin complexes [30]. Resonance Raman data for iron porphyrin complexes indicate that core expansion and the out-of-plane displacement of the iron are unrelated, at least for the ferric high-spin hemes which are typically found in these heme proteins [9]. The thermal spin conversion process would then be expected to be accompanied by substantial structural changes at the heme, although the changes cannot be identified beyond the bond length expansion, as the spin on the $S = 1/2$ iron increases to $S = 5/2$. The hemin-N_3-dimethyl sulfoxide complex is a simple azide-heme system that exhibits spin equilibrium with a $T_c = 140$ K [32]. This equilibrium leads in the extreme, to the complete rupture of the Fe(III)–dimethyl sulfoxide oxygen bond, to produce a five-coordinate, high-spin hemin-azide complex. Several iron(III) porphyrin complexes with azide have been prepared which suggest that the spin equilibrium is primarily determined by the ligand that is *trans* to the azide [32], or in the case of catalase, *trans* to the phenolate amino acid.

Complexes between beef liver catalase and azide, isothiocyanate or isocyanate are not unique in converting only incompletely to a low-spin species, even at very low temperatures [15,33], for the equilibrium may appear to be temperature independent below a specific temperature, T_o. The residual paramagnetism in inorganic complexes of iron(II) has been associated with a lattice effect in which a given lattice may be expected to have only a limited tolerance for change in the intramolecular dimensions which accompany the spin-state transition. Similarly, it has been suggested that in frozen solutions the globin and surrounding ice become too rigid to accommodate the changes in bond lengths which accompany the spin change, so that a random mixture of high- and low-spin hemes becomes frozen in, and a true thermal equilibrium cannot be attained. For example, the T_o value for oxycobalt-myoglobin is the freezing point of the solvent. Above T_o there appears to be only one configuration for the molecular structure of the complex, whereas at 77 K, well below T_o, two distinct orientations of oxygen bound to the heme have been observed [34]. The catalase data clearly indicate that protein structural changes are not simply limited to the freezing point of the solvent but are also dependent on the protein structure itself, and may occur at very low temperatures.

The observation of large temperature-dependent changes in the absorption spectra of complexes of catalase depends primarily on the intermediate ligand field strength of the axial ligand coordinated to the ferric heme iron. The temperature dependence can be explained as arising from a thermal spin conversion at very low temperatures. These data suggest that significant, but limited, structural changes can occur in glassy protein solutions at very low temperatures. This interpretation suggests that subtle electronic or structural changes may occur at the metal-axial ligand binding sites which do not necessarily give rise to spin changes, but are the result of the temperature-dependent conformational changes in the protein. The effects of these conformational changes on the analysis of spectral data may be especially significant in the comparison of results obtained from techniques using widely differing sample temperatures, and which attempt, in the end, to describe the mechanism of room-temperature biochemical reactions.

Acknowledgement

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