

Stabilization of Catalase-Ammonia Complex in Frozen Solution

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It is demonstrated that the ammonia complex of catalase from *Micrococcus lysodeikticus* is difficult to detect in liquid solution but easy to study in frozen solution. Electron spin resonance and light absorption of catalase and the ammonia complex have been measured in frozen solutions down to 77°K. Factors that possibly could effect a stabilization of the ammonia complex in the matrix of ice are discussed.

Since the days of Kamerlingh-Onnes it has been common in physics to make measurements at low temperatures. One reason for this is that many physical parameters depend on temperature in such a way that at low temperatures accuracy in their determination is greatly enhanced. Some phenomena and transitions are possible to detect only at very low temperatures, as for instance superconduction.

Also in physical biochemistry it has become obvious that measurements at low temperatures are of great value. Keilin and Hartree discovered¹ that in frozen solutions of cytochrome c, in its reduced state, the light absorption bands when measured at low enough temperature were greatly intensified, sharpened, and split into several bands. This phenomenon has been used extensively in the investigation of various heme proteins.²

Measurements of ESR (electron spin resonance) at low temperatures offers a number of advantages. The dielectric losses in aqueous solutions are very much smaller when the solutions are frozen. Hence, sensitivity of the measurements is greatly enhanced. Sensivity is also gained upon lowering the temperature since the absorption intensity varies over wide ranges of temperature roughly proportional to the inverse of the absolute temperature. Further more, the temperature dependence of relaxation phenomena sharpens many absorption lines with decreasing temperature. Hence myoglobin has been investigated at low temperatures in the crystalline form³ and in frozen solutions.⁴ In xanthin

oxidase for instance, the ESR absorption of the iron is difficult to observe if the temperature is not low enough.⁵

By freezing it is possible to stabilize an unstable paramagnetic species or to stop a reaction at a desired stage and investigate the sample at leisure.⁶ For this purpose a rapid freezing technique has been developed by Bray⁷ and applied to the reaction of xanthin oxidase with great success.⁵

The Mössbauer effect in heme proteins enriched with ⁵⁷Fe has very recently been investigated.⁸ These measurements must be made in solids, *e.g.* in frozen solutions where recoil free absorption can take place.

It is obviously of great importance for the interpretation of these various results to understand to what extent the results obtained at low temperatures are representative of the system under investigation, when it is in a liquid aqueous solution or in the living cell.

Redox equilibria and ionizations are known to change with temperature. For the respiratory chain it is known that the temperature coefficients (measured around room temperature) of the intercarrier reactions are about equal, so that the steady states do not change appreciably upon freezing.⁹ However, the situation might be altered at the temperatures of liquid N₂ and liquid He, since electron transfer reactions may still take place at very low temperatures. Conformational changes during freezing should also be considered.

During the course of our investigations of the temperature dependence of the ESR absorption of heme proteins, we have found an anomalous behaviour of catalase in ammoniacal solution, which will be reported below.

MATERIAL AND METHODS

Micrococcus lysodeikticus was grown in large quantities according to Beers.¹⁰ Catalase was prepared from the bacteria according to the method described by Herbert and Pinsent¹¹ with modifications introduced by Brill and Maehly.¹² For the final catalase sample the index A_{405}/A_{280} was found to be 0.83. *Kat. F* was determined as 145 000.

The ESR measurements were made with a Varian X-band spectrometer with Fieldial, 100 kc field modulation and liquid nitrogen dewar insert.

Low temperature light absorption spectra were obtained with the recording spectrophotometer described elsewhere.² A light path of 2 mm was used.

RESULTS AND DISCUSSION

Bacterial ferricatalase in phosphate or EDTA buffer devoid of ammonia gives, in frozen solution at low temperature, an ESR-absorption with two intense peaks at low fields, $g \approx 6.2$ and $g \approx 5.8$; see Fig. 1.

Ferriporphyrine derivatives with ESR absorption of this kind are known to be high spin compounds.^{3,4} The observation, that there are two peaks around $g = 6$ indicates, that there is a distorted axial symmetry of the heme and/or that all four heme groups of the catalase molecule are not identical.

The frozen solution of high spin ferricatalase is green. When small amounts of ammonia are added to the catalase solution its light absorption at room temperature is not altered to any visible extent. Upon freezing, however, the sample now converts into a brick red colour. Also the ESR absorption changes

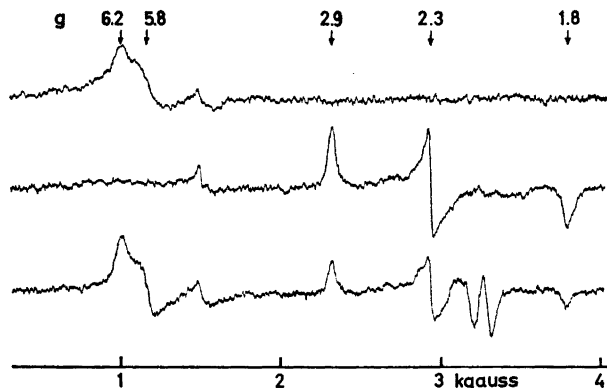


Fig. 1. ESR first derivative recordings at 77°K of *M. lysodeikticus* catalase in 80 mM EDTA (upper curve) and after addition of 100 mM NH_3 (middle curve). Unbuffered catalase after addition of a small amount of NH_3 (lower curve). In the last case contaminating Cu^{2+} had not been removed by dialysis with EDTA, hence the structure of the absorption around 3 kgauss. Catalase iron concentration was 835 μM .

dramatically as shown in Fig. 1. The characteristics of a high spin ferriheme compound have disappeared. Instead there are three g -values at approximately 2.9, 2.3, and 1.8. A great number of low spin ferriporphyrin compounds give at 77°K ESR absorptions with three principal g -values centered around $g = 2$. The low spin compound described here is presumably a ferricatalase ammonia complex. Ammonia compounds have been detected by their light absorption in aqueous solution for ferrihemoglobin and ferrimyoglobin and shown to be of the low spin type.^{13,14}

With low enough concentration of the added ammonia it was possible to create conditions where there was only partial formation of the ammonia

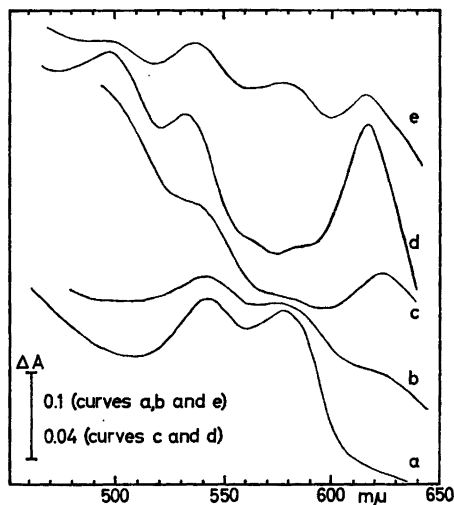


Fig. 2. Light absorption at low temperature of *M. lysodeikticus* catalase samples according to Table 1. Concentration of catalase iron was 85 μM . a: Sample II (18 mM NH_3 , pH 10.3, 77°K). b: II warmed but still frozen (243°K) c: II thawed (283°K). d: Sample V (no NH_3). e: Sample IV (0.74 mM NH_3 , pH 9.7, 77°K).

complex. This is demonstrated by the bottom tracing of Fig. 1 where a mixed type ESR spectrum is shown. With low temperature spectrophotometry, which needs less material, some further details of the requirements for the temperature dependence of the catalase-ammonia complex formation have been investigated. The results are summarized in Fig. 2 and Table 1.

In phosphate buffer at pH 8.9 and 10.4 the spectrum is typical for that of a high spin ferricatalase compound (*e.g.* catalase or catalase fluoride in liquid aqueous solution) with absorption maxima at 617, 533, and 497 $m\mu$. After addition of ammonia the absorption was converted into a low spin type spectrum, with maxima at 578 and 543 $m\mu$, similar to that of, *e.g.* catalase cyanide.

At pH 9.7 the formation of the ammonia complex in the frozen solution was still practically complete using 1.5 mM free NH_3 . With 0.74 mM free NH_3 only partial formation of the complex took place. At pH 7.9 a partial complex formation was obtained with 21 mM free NH_3 . It is evident that the concentration of unprotonated NH_3 is one decisive factor in the formation of the complex. This confirms our hypothesis that the low spin compound is a catalase to ammonia complex.

With two samples (II and IV, of Table 1) the liquid N_2 of the low temperature dewar was poured out and the cuvettes were left to slowly rise in temperature. The sample temperature was followed with a thermistor fixed to the cuvette and coupled to a calibrated bridge. In both cases the ratio between free catalase and the ammonia complex did not change until just below or at the thawing point when all but perhaps a trace of the complex disappeared. This confirms our visual observation with the ESR experiments, *i.e.* the ammonia complex is formed during the process of freezing the sample.

Our data do not permit any calculation of a dissociation constant for the ammonia complex. The meaning of a dissociation constant in the frozen sample would also be somewhat doubtful. It is, however, clear that it is at least about 100 times easier to form the complex in frozen solution than in liquid solution since 18 mM NH_3 at pH 10.3 at room temperature gives no complex or only a trace of it, whereas 1.5 mM NH_3 at pH 9.7 gives practically complete complex formation in frozen solution.

A similar type of transition on freezing has been described by Keilin and Hartree¹ for the conversion of alkaline methemoglobin to acid methemoglobin. They suggested that the associated spectral change was due to a shift in the equilibrium of water and hydroxyl ion associated with the sixth coordination position of the iron atom resulting from a suppression of the ionization of water at low temperatures.

In the present case it is concluded that the catalase-ammonia complex is greatly stabilized in the matrix of ice. The molecular basis of this stabilization is not known. An enhanced deprotonation of NH_4^+ at freezing is not likely to be the sole cause of stabilization since at pH 10.3 not more than 20 mM NH_3 gives complete formation, whereas at pH 7.9 more than 21 mM gives only partial complex formation. A shift at freezing of the ionization equilibrium of some group or groups in the catalase protein affecting the heme or its immediate surrounding could possibly be a factor stabilizing the complex.

Table 1. Conditions and spectral type for some of the light absorption spectra recorded.

Sample	Cat Fe μM	concentrations			pH	Temp. $^{\circ}\text{K}$	Spectral type
		Buffer Component mM	$C_{\text{NH}_4} + C_{\text{NH}_3}$ mM	C_{NH_3} mM			
I	85	K_2HPO_4 20 KOH 1	0	0	10.4	77	High spin
II	85	K_2HPO_4 20	20	18	10.3	77	Low spin
» warmed but still frozen	»	»	»	»	»	243	Low spin (+ trace of high spin?)
» thawed	»	»	»	»	»	283	High spin (+ trace of low spin?)
III	85	K_2HPO_4 20 KOH 1	2	1.5	9.7	77	Low spin + trace of high spin
IV	85	K_2HPO_4 20 KOH 1	1	0.74	9.7	77	Mixed spin
» warmed but still frozen	»	»	»	»	»	257	Mixed spin
» thawed	»	»	»	»	»	278	High spin (+ trace of low spin?)
V	85	K_2HPO_4 20	0	0	8.9	77	High spin
VI	21	KH_2PO_4 70	100	15	8.5	77	Mixed spin (most low spin)
VII	21	KH_2PO_4 750	500	21	7.9	77	Mixed spin (most high spin)
VIII	21	KH_2PO_4 1000	1000	11	7.3	77	High spin

It is very likely that the steric requirements of the matrix of ice freezes the protein into a certain conformation with a structure at the heme where ammonia fits in. This is the same as saying that catalase exists in more than one allosteric modification. One of these modifications has a high affinity for ammonia but it exists only to a very small fraction in the liquid solution at room temperature. In the matrix of ice this modification is stabilized and if ammonia is available the complex is formed upon freezing. Monod *et al.*¹⁵ have recently discussed the possibility of equilibria between allosteric modifications as the cause of the sigmoid oxygenation curve of hemoglobin as well as of the shapes of the activity curves of various enzymes. Further experimental information will be necessary in order to test the validity of the hypothesis in the present case.

Whatever the molecular basis may be for the stabilization of the ammonia complex of catalase in frozen solution, the present investigation demonstrates a case where it is hardly possible to detect the complex in liquid solution but very easy to study it in frozen solution. Similar transitions may occur when enzymes, enzyme systems, microsomes or mitochondria are investigated in frozen solutions. Even if it is still possible to draw conclusions concerning the redox equilibrium in the frozen sample, the formation of the complex *per se* might possibly cause a shift of this equilibrium.

Acknowledgements. This work was supported in part by grants from the *National Institutes of Health*, USA (AM-05895 and GM-12 202). One of the authors (A.E.) wishes to thank Dr. Britton Chance for hospitality during his stay in Philadelphia. This study was carried out during the tenure of a U.S.P.H.S. Research Career Development Award (GM-K3-4111) to one of us (R.W.E.).

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Received March 14, 1966.