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# Temperature-Dependent Denaturation States of Horse and Human Ferro- and Ferrihemoglobins*

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ABSTRACT: The conformational differences that have previously been shown to exist between horse ferrihemoglobin ($Hb^+$) denatured by acid at 0 and 25° have been confirmed by optical difference spectra. Similar optical differences are exhibited by human $Hb^+$ and, as with horse $Hb^+$, occur only at those wavelengths (*ca.* 370 nm) at which dimerized heme manifests its presence. Carbonylhemoglobin also exhibits a temperature-dependent difference spectrum when the denaturation pH is 1.95, but not at pH 3.2. In combination with previously published data, these results imply that separation of the prosthetic group is a prerequisite for optical observation of the difference in the denatured apoprotein at the two temperatures.

It has been known for some time that ferrihemoglobin denatured by acid at 0° differs from the denatured protein formed at 25° (Beychok and Steinhardt, 1960). This conclusion was based on a detailed analysis of the large differences in intrinsic viscosities of the proteins denatured at the two different temperatures, and of the effects of ionic strength and temperature on the titration curves of the two products.

It has also been shown that the apparent energy of activation determining the denaturation rates is much smaller below 12° than above this temperature with both horse (Beychok and Steinhardt, 1960) and human (Steinhardt and Hiremath, 1967) ferrihemoglobin; a difference in the reaction paths and, therefore, in the products formed at 0° and 25° is thus suggested. Allis and Steinhardt (1969) showed that the temperature coefficient of the simultaneous unfolding of the protein and separation of the heme changes rather than that of the subsequent dimerization of the heme.

In the present investigation, we have sought to further characterize these differences by a close study of differences in the absorption spectra of the denatured products. The purpose was to contribute to an understanding of the molecular basis of the difference between the products since this difference might illuminate the nature of the interactions involved in forming the tertiary structure of the native protein.

## Experimental Procedure

*Materials.* Horse COHb was crystallized three times from the blood of a single animal using the method of Ferry and Green (1929) as described earlier (Steinhardt *et al.*, 1966).

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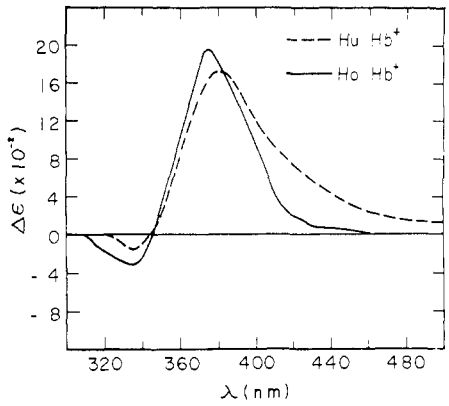


FIGURE 1: Ferrihemoglobin difference spectra of the denaturation product at 0° *vs.* the denaturation product at 25°. The final pH is 2.65 and all measurements are made at 0°.

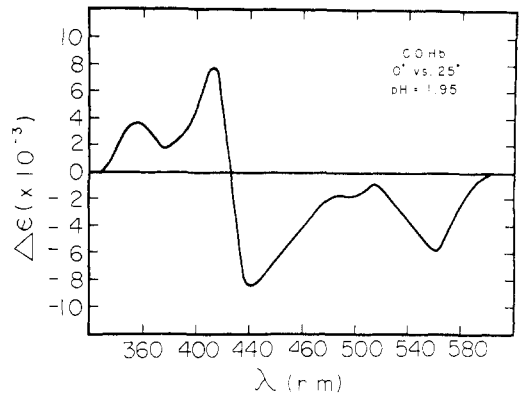


FIGURE 2: Horse carbonylhemoglobin (COHb) difference spectrum of the denaturation product at 0° *vs.* the denaturation product at 25°. All measurements are made at 0°.

Human COHb was also crystallized three times from pooled human red blood cells by the procedure of Drabkin (1949) as previously described (Steinhardt and Hiremath, 1967). Both proteins were stored frozen in 5% solutions. Two preparations of horse COHb and one preparation of human COHb were used; their molar extinction coefficients were 185,000–190,000 at 419 nm, 12,500–14,500 at 538 nm, 12,200–13,700 at 569 nm. The absence of detectable peaks at 500 or 630 nm indicated that the ferri form was not present.

The preparations were oxidized to $Hb^+$ in 2% solutions with 1.5 equiv of $K_3Fe(CN)_6$ (Steinhardt and Hiremath, 1967) and afterward were dialyzed against 0.2 M phosphate buffer (pH 6.9) followed by dialysis against distilled water. The extinction coefficients of human $Hb^+$ and horse $Hb^+$, respectively, at 405 nm were 165,000–170,000 and 170,000–175,000.

Reagent grade chemicals were used without further purification.

*Methods.* $Hb^+$. Two solutions of ferrihemoglobin were acidified with HCl so that the resulting concentrations (0.085%), ionic strengths (0.02 M $Cl^-$), and pH (2.65) were identical. One was denatured at 0° in a cold room for seven half-periods, the other at room temperature (23°) for the same length of time. Both samples were examined by difference spectroscopy in 5-mm absorbance cells in a Cary 14 spectrophotometer equipped with thermostated cell holders (0°). Spectral differences between the two samples were recorded using an expanded scale (0–0.1 o.d.) slide-wire.

COHb. The experimental procedure for COHb denatured at pH 1.95 was essentially the same as that above except that oxygen was scrupulously excluded from the denatured ferroprotein due to its ease of oxidation. This precaution was accomplished by using tonometers and 0.5-mm absorption cells with long necks as previously described (Steinhardt *et al.*, 1966). Protein concentrations were increased (0.3–0.4%) to minimize the effects of residual oxygen and all solutions were saturated with carbon monoxide before use. A regular (0–1.0 o.d.) slide-wire was used for both the difference spectra and the direct spectra.

At pH 3.2, the spectral differences encountered with COHb were so small that even slight concentration differences could not be tolerated. Therefore, denaturation was carried out in the cold room in a single tonometer with two long-necked cells attached to it. The spectra of the samples thus prepared differed only slightly from each other (see Results) due to small path-length differences in the cells.

## Results

*$Hb^+$.* Figure 1 shows the difference in absorption between protein denatured at 25° and that denatured at 0° with both horse and human ferrihemoglobin when both are measured at 0°. In those regions of the spectrum not shown (both farther ultraviolet and visible) there is no difference spectrum, *i.e.*, the direct spectra of the two products are identical there.

After the difference spectrum had been obtained, the sample denatured at 0° was allowed to stand at room temperature for approximately 2 hr. After this time, it was placed back in the cell compartment, equilibrated to 0°, and again its absorbance against the sample denatured at 25° was measured. In all cases the spectral difference previously observed had disappeared completely. This observation accords with the finding of Beychok and Steinhardt (1960) that the protein denatured at 0° could be rapidly converted into the protein denatured at 25° by simply elevating the temperature. If, however, the denaturation were carried out at 25° in the absence of buffers, the product would maintain its identity despite extended periods of incubation at 0°.

*COHb.* At pH $\leq$2.0 heme is separated from carbonylhemoglobin (Allis and Steinhardt, 1970) just as in ferrihemoglobin at higher pH (pH 3–4). The experimental difference spectrum obtained at pH 1.95 is shown in Figure 2. Although the spectral differences are more extensive than those of the ferrihemoglobins (Figure 1), they completely disappear when the 0° product is warmed and reexamined at 0°. This finding implies that rates of conversion from one product to another are of the same magnitude for COHb as for $Hb^+$.

Direct spectroscopy of the products individually yielded the results in Figure 3. Unlike the case of ferrihemoglobin, there is considerable spectral difference between the two carbonylhemoglobin denaturation products in the visible region. However, the ultraviolet spectra of the two were identical.

When COHb was denatured at pH 3.2 the difference spectra were too small to be detected by the techniques employed above. It was extremely difficult, therefore, to eliminate the interfering effects of slight concentration differences due to evaporation in one or the other tonometers, optical path-length differences, and most importantly, oxidation. All of these problems were encountered with COHb at pH 1.95 but could be reduced to an acceptable level relative to the large spectral differences encountered there.

By employing the tandem vessel described under Methods,

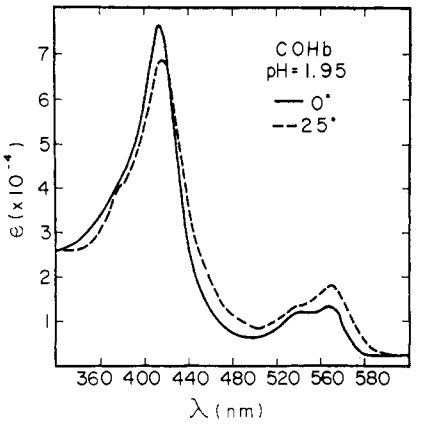


FIGURE 3: Direct spectra of horse COHb denatured at 0° and at 25°. All measurements are made at 0°.

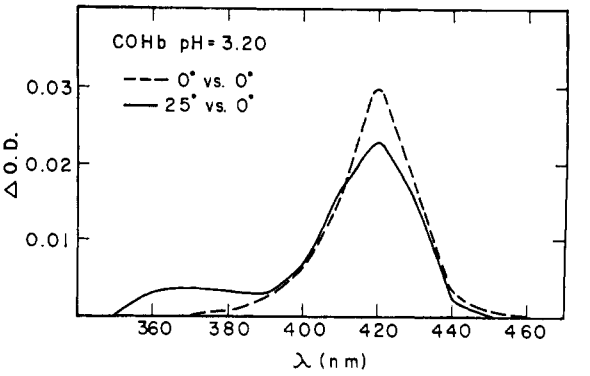


FIGURE 4: Difference spectra of horse COHb denatured at 0° *vs.* an identically prepared sample (---) and horse COHb denatured at 0°, warmed to 25° for 2 hr, and rerun *vs.* the original reference (—). Protein concentration is 0.31%, optical path length is 0.5 mm, chloride ion concentration is 0.02 M, pH is 3.20, and the temperature is 0°.

it was possible to prepare two identical samples, both denatured at the low temperature. Reference to Figure 4 shows that there was a slight spectral difference between the two, caused primarily by a very small optical path-length difference in the absorption cells.

One of the two cells was allowed to stand at room temperature for 2 hr on the assumption that if there were a difference between carbonylhemoglobin at pH 3.2 denatured at 0° and the protein denatured at 25°, the 0° product would transform to the species which would have been formed had the denaturation occurred at 25° on incubation at that temperature. This assumption is supported by the behavior of denatured $Hb^+$ and COHb at pH 1.95 where such a transformation was observed to occur.

When COHb originally denatured at 0° and pH 3.2 was warmed, no change occurred. The small differences which do exist are the result of a very slight oxidation of the warmed species. The conclusion to be drawn from Figure 4 is that the absorption spectrum of COHb denatured at pH 3.2 and 0° is in all respects identical with that of the room temperature denaturation product.

## Discussion

Polet and Steinhardt (1969) have shown that when $Hb^+$ is denatured, heme, after being expelled from its pocket, dimerizes and attaches to the apoprotein at a site different than the one it occupies in the native protein. This occurrence is attended by the appearance of a broad band in the spectrum centered at 370 nm. Because the two temperature-dependent products of horse and human $Hb^+$ differ spectroscopically only in this same spectral region (Figure 1), it appears that the protein denatured at 25° and that denatured at 0° differ in the environment of the dimerized heme at its point of attachment to the unfolded apoprotein. Other differences, which do not give rise to changes in extinction coefficients are, of course, not excluded. The heme furnishes a signal that the conformations formed at 0° and 25° are not the same at at least one point. However, the absence of a difference spectrum in the 270- to 290-nm region also excludes differences in conformation that would affect aromatic side chains.

It was to be expected that carbonylhemoglobin denatured at pH 3.2 would not yield results similar to those with ferrihemoglobin. Allis and Steinhardt (1970) have shown that although COHb is completely acid unfolded at all pH values below 3.1, the heme is not expelled from all of the protein molecules at any pH above 2.0. Accordingly, we observe (Figures 2 and 3) an expected difference spectrum at pH 1.95, but not at pH 3.2 (Figure 4).

Further implications of the spectral differences can be better appreciated by inspecting the direct spectra of the products of COHb acid denaturation formed at pH 1.95 (Figure 3). The higher Soret peak found at 0° is more nearly typical of denaturation at pH values above pH 2 and lacks the 380-nm shoulder characteristic of dimeric ferroheme (Figure 1 in Allis and Steinhardt, 1970), as if at the lower temperatures dimerization of the ferroheme were diminished. This phenomenon is compatible with the hypothesis that CO ferroheme is not fully separated from the apoprotein at 0° and is therefore unable to dimerize. However, if the only effect of the lower temperature were the displacement of the equilibrium between the bound and unbound CO ferroheme to lower pH values, the Soret maximum would be expected to occur at 418 nm (Figure 1 in Allis and Steinhardt, 1970); in fact, it appears at 412 nm (Figure 3). Thus, the 0° situation is not fully clear. At a minimum, however, it is clear that COHb denatured at pH 1.95 at 0° differs from the denaturation product at 25°. In this there is a clear parallel with the results obtained with $Hb^+$ where heme separation and dimerization have been demonstrated (Polet and Steinhardt, 1969). It must be concluded that at pH 1.95 and 0° there exist in denatured COHb other differences than those previously discussed, *i.e.*, a difference in the aggregation state of the CO heme, or residual interactions of the latter with the apoprotein which are qualitatively different from those which operate in $Hb^+$ or in COHb at 25°.

The lack of a temperature effect on the spectrum of denatured COHb at pH 3.2 confirms that heme detachment is a prerequisite for optical observation of the difference in the denatured apoprotein, at the two temperatures. Since, at pH 3.2, all of the heme remains attached to its original position, no spectral differences should occur between the denaturation products at 0° and 25°, in accordance with Figure 4. However, the unfolded protein denatured at 0° and 25° at pH 3.2 have not been shown to be identical. Rather, heme attached to the apoprotein cannot function as a spectral probe as it does in the case of $Hb^+$ at more acid pH values. Again, the absence of a difference spectrum at 280 nm eliminates conformational differences involving the aromatic amino acids as well, but other changes in conformation may exist.

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# Antigenicity of Polypeptides (Poly-α-amino Acids). Physicochemical Studies of a Calcium-Dependent Antigen–Antibody Reaction*

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ABSTRACT: The precipitation of a population of homologous sheep antibodies against α-poly(Glu$^{60}$Ala$^{30}$Tyr$^{10}$) (GAT) has been shown previously to specifically require calcium ions. The effect of $Ca^{2+}$ on the interaction between the "calcium-dependent" antibody and GAT has been studied by ultracentrifugation, the use of [$^{14}$C]GAT in combination with rabbit anti-sheep γ-globulin, and an immunoabsorbant containing GAT. No interaction occurs unless $Ca^{2+}$ is present. Calcium has no effect on the sedimentation coefficient, hydrogen-exchange kinetics, or fluorescence emission spectra of purified calcium-dependent antibody. In the presence of 0.01 N $Ca^{2+}$, $s^0_{20,w}$ of GAT increases slightly, [η] decreases by 39%, and optical rotatory dispersion shows small changes. These data all indicate a more compact configuration. A correlation between the ability of $Ca^{2+}$ to induce configurational changes in glutamic acid containing polypeptides and their respective ability to induce calcium-dependent antibody in sheep antisera has been shown. The ability of divalent ions to cause interaction between GAT and calcium-dependent antibody, *i.e.*, ($Ca^{2+} > Sr^{2+} > Mn^{2+} > Ba^{2+} > Mg^{2+}$) corresponds to the order of divalent ion binding to GAT. In addition, when $Ca^{2+}$ is added in increments to calcium-dependent antibody mixed with excess GAT (incomplete antigen), the resultant precipitin curve has the same shape as would be expected were increasing amounts of antigen added to its respective antiserum. These data lead to the conclusion that $Ca^{2+}$ induces unique conformational determinants in glutamyl containing polypeptide antigens. The optical rotatory dispersion data and other considerations preclude the possibility that $Ca^{2+}$ induces helical segments in these antigens.

This, plus the fact that monovalent cations are ineffective, lead to the conclusion that $Ca^{2+}$ acts mainly by bridging two glutamyl carboxyls.

Recently (Maurer *et al.*, 1970) we reported a study of dilution and specific ion effects on the precipitin reaction of several sheep and rabbit antisera directed against synthetic polymers of amino acids. The data presented demonstrated that sheep and rabbit produce two populations of antibody (termed calcium-dependent and calcium-independent antibody) against a random sequence polypeptide,[1] α-(Glu$^{60}$-Ala$^{30}$Tyr$^{10}$)$_n$ (GAT), where the precipitin reaction of one of these populations has a specific requirement for calcium ion. Three areas where calcium might have its effect on the precipitin reaction are (1) on a "preexisting" soluble antibody–antigen complex, *i.e.*, the role of calcium as a nonspecific insolubilizing agent; (2) on the antibody; and (3) on the conformation of the antigen, GAT (a highly negatively charged polypeptide). This paper is a report of results of physicochemical studies designed to determine the role of calcium in the precipitin reaction of the calcium-dependent anti-GAT.

In an attempt to further elucidate gross differences in the antigenic determinants of GAT with respect to calcium-dependent and -independent antibodies, we have studied the effects on the precipitin reaction of limited chemical modifications (methylation) of the glutamyl residues of the polypeptide antigen.

## Materials and Methods

The preparation, analysis, and handling of antisera, and the purification of calcium-dependent antibody have previously been described (Clark and Maurer, 1969; Maurer *et al.*, 1970). The preparation and properties of the various

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[1] Polypeptide nomenclature used is based upon recommendation of IUPAC-IUB Commission (1968) on Biochemical Nomenclature, Oct 1967. In addition, abbreviations used are: GAT, α-poly(Glu$^{60}$Ala$^{30}$Tyr$^{10}$); GA, α-poly(Glu$^{60}$Ala$^{40}$); BBS, sodium borate (0.01 M) buffered saline (pH 7.5); MBSA, methylated bovine serum albumin; G$^{60}$A$^{40}$-amide, poly(α-*N*-3-hydroxypropyl-α-glutamide $^{60}$Ala$^{40}$).