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On the Thermodynamic Basis of Induced Fit. Specific Alkane Binding to Proteins*

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ABSTRACT: Small alkanes bind to β-lactoglobulin, ferrihemoglobin, and ferrimyoglobin, in each case, to a localized, interior, hydrophobic site with high affinity and nontrivial stereospecificity. In order to understand this behavior, the thermodynamic parameters for the transfer of pentane, neopentane, and butane to water, from ideal solutions, dodecyl sulfate micelles, ferrimyoglobin, ferrihemoglobin, and β-lactoglobulin, between 0 and 40° or 50°, were determined. ΔC_p for the proteins, and for all solutes, is comparable with the dodecyl sulfate values; this variation of ΔH with temperature derives from the unusual behavior of water, and is typical of "hydrophobic" interactions. The heat of dissociation of butane and pentane from β-lactoglobulin and ferrihemoglobin is 3–4 kcal higher than from dodecyl sulfate micelles over the whole temperature range, and is the source of the high alkane affinity of these proteins. For neopentane, ΔH for β-lactoglobulin is more negative, and for ferrihemoglobin more positive, by 1–2 kcal, than the dodecyl sulfate values: β-lactoglobulin makes a clear distinction between pentane and neopentane, while ferrihemoglobin does not. The excess ΔH cannot arise from the solute–water interaction, from a nonoccurring displacement of water, or from exceptionally strong solute–protein interactions, but must come from suboptimal interactions in the unoccupied site (plausibly, abnormally large molar and "free" volumes), which are normalized by strictly local rearrangements in the butane and pentane, but not (for β-lactoglobulin) the neopentane, complexes.

The origin of the high affinity and dramatic stereospecificity of enzymes for their substrates is a major problem of biochemistry. Whatever general model one assumes, any particular active site must, of course, still be constructed to interact with the substrate in modes dictated by the structure of the substrate: monopole or dipole attractions, London interactions, hydrogen bonding, covalent bonding, or whatever. In the classical "lock and key," or template, model, appropriately complementary groups are rigidly positioned for maximal interaction with the substrate, and no other, molecule; it is usually implicit that the shape and nature of the cavity will provide good, close, van der Waals contacts. The favorable local free energies sum to the overall free energy of binding. Koshland (see Koshland and Neet, 1968) has long proposed that the complete active site may arise only by an "induced fit:" in the act of binding, the enzyme structure is modified to achieve the interactions required by the specific substrate. It is not required, but it is often implicit, that the changes be large: for example, the known shift of tyrosine-248 when glycyltyrosine is bound to carboxypeptidase A (Lipscomb *et al.*, 1968) and the speculative postulated rearrangement of β-amylase (Koshland *et al.*, 1962). It also seems to be assumed that, since the new configuration is not observed in the absence of substrate, the contribution of the rearrangement itself to the overall free energy of binding must be positive (*i.e.*, unfavorable). In both models the native enzyme tends to be thought of as a well-behaved, stable structure, not only generally, but locally. I would like to add a third model, for which experimental examples are given below, in which the active site is so constructed that the free energy of binding of the right substrate is more negative than one thought one had the right to expect.

Alkanes bind to β-lactoglobulin (Wishnia and Pinder, 1966), ferrimyoglobin, and ferrihemoglobin (Wishnia, 1969), in each case, to a localized, interior, strictly hydrophobic site, with high affinity and nontrivial stereospecificity. For this type of interaction where exist explicit normative models for maximal, ideal, interaction: transfer of alkanes from aqueous solution to liquid alkanes (see, *e.g.*, Kauzmann, 1959) or, for convenience, to the interior of detergent micelles (Wishnia, 1963). The thermodynamic parameters to be expected for the progressively weaker interactions of alkanes with solvents of increasing polarity (*e.g.*, hexane, benzene, and dioxane, Thomsen and Gjaldbaek, 1963; methanol, ethanol, and 2-propanol, Kretschmer and Wiebe, 1952; among others) are also known. Since the binding of some alkanes to βLG,[1] Hb^+, and Mb^+ is, in fact, *stronger* than to quasi-ideal systems like dodecyl sul-

* Contribution from the Department of Chemistry, State University of New York at Stony Brook, Stony Brook, New York 11790. *Received July 24, 1969.* Supported by funds from National Science Foundation Grant GB-5596.

[1] Abbreviations used are: βLGA, β-lactoglobulin A; βLGA-1, βLGA monomer at pH 2.0; βLGA-2, βLGA dimer at pH 5.3; Hb, deoxyhemoglobin; Hb^+, ferrihemoglobin; Hb^+CN^-, ferrihemoglobin cyanide; HbO_2, oxyhemoglobin; Mb^+, ferrimyoglobin; and ApoMb, apomyoglobin.

TABLE I:[a] The Coefficients of $\Delta G = A + BT + CT^2 + DT^3$.

	A	B	C	$D \times 10^4$	σ (cal)
Pentane					
Ideal	−20,113	185.23	−0.42426	3.5407	26
Dodecyl sulfate	−1,969	−14.96	0.29334	−5.2171	14
Mb^+[b]	−7,984	50.56	0.08409	−3.0803	30
Hb^+	−4,763	41.39	0.07898	−2.8670	17
βLGA-1	−11,312	88.19	−0.00738	−2.5786	28
βLGA-2	−1,644	−5.93	0.30304	−6.0090	19
ApoMb	−13,544	53.99	0.29188	−8.0283	12
HbO_2	−307	17.85	0.08850	−2.2501	23
Hb^+CN^-	−1,916	27.79	0.07508	−2.3097	20
Hb^+	−4,077	32.59	0.12775	−3.7673	31
Hb	−13,067	69.89	0.16713	−5.8740	16
Butane					
Ideal	−8,662	60.85	0.00007	−1.3593	13
DS	11,658	−138.73	0.64180	−8.4291	14
Mb^+[b]	0	−14.04	0.21438	−3.6089	57
Hb^+[b]	−5,681	44.60	0.03705	−1.8798	33
βLGA-1[b]	−8,474	91.02	−0.15164	0.4875	25
βLGA-2[b]	3,577	−29.93	0.25975	−4.2259	24
Neopentane					
Ideal	5,652	−75.71	0.44900	−6.2186	12
DS	−6,998	36.38	0.11511	−3.2410	11
Hb^+[b]	−907	3.77	0.15111	−3.0063	47
βLGA-2	−9,177	66.43	−0.01502	−1.4253	86

[a] Temperature ranges: ideal, 0–50°, dodecyl sulfate, 10–50°, ApoMb, 0–30°, and other proteins, 0–40°. The coefficients reproduce the data well, but have no intrinsic interest: successive runs which yield quite good agreement among ΔG, ΔH, etc., can produce wildly different coefficients. [b] K_0 used.

fate micelles (Wishnia, 1963) or bovine serum albumin (Wishnia and Pinder, 1964), but with unexpected differences in the behavior of homologous compounds, it seemed useful to study the thermodynamics of binding in detail. Therefore, ΔG, ΔH, ΔS, and ΔC_p for the transfer of pentane, neopentane, and butane to water, from ideal solutions, dodecyl sulfate micelles, Mb^+, Hb^+, and βLGA, at 5° intervals in the range 0–50°, were determined.

Experimental Section

Bovine β-lactoglobulin A, human hemoglobin, and sperm whale myoglobin solutions were prepared as described previously (Wishnia and Pinder, 1966; Wishnia, 1969). Purification of [^{3}H]alkanes, determination of specific activities, and measurement of alkane solubility or binding have also been described (Wishnia and Pinder, 1966).

The free energies of the transfer of alkane from nonpolar to aqueous environments were calculated as follows: ΔG (ideal), from $\Delta G = -RT \ln (Xf^0/f)$, where X is the observed mole-fraction solubility in water at the low partial pressure p, and f and f^0 are the fugacities at p and the saturated vapor pressure p^0 calculated from Dreisbach (1959); ΔG (dodecyl sulfate), from the mole fraction partition between aqueous and micellar "phases." Alkane partial pressures were kept low and adjusted to keep the mole-fraction in the micelles roughly constant. The different temperature dependence of ideal and micellar parameters may be real (perhaps from changes in micellar size with temperature), or might include some systematic error in determination of the experimental or calculated pressure of the alkanes.

ΔG (protein) was calculated from the mole-fraction dissociation constants. Extensive studies at 0 and 25° showed that binding falls into two classes: $K_2 \gg K_1$ and $K_2 \sim 4K_1$. In the first class only K_1 was studied; in the second class it was *assumed* that $K_2 = 4K_1$ and the curves fitted for two noninteracting equivalent sites with the same intrinsic K_0. In this way, we hope to deal with true microscopic, rather than phenomenological, constants, although in the cases we will discuss the distinction is of little consequence.

The experimental values of ΔG were fitted to a cubic polynomial in absolute temperature, $A + BT + CT^2 + DT^3$, by standard least-squares procedures (Margenau and Murphy, 1956). For the record, the coefficients, the temperature range studied, and the root-mean-square error in ΔG are given in Table I. ΔH, ΔS, and ΔC_p were calculated from appropriate derivatives ($\Delta S = -d\Delta G/dT = -B - 2CT - 3CT^2$, $\Delta H = \Delta G + T\Delta S = A - CT^2 - 2DT^3$, $\Delta C_p = d\Delta H/dT = -2CT$

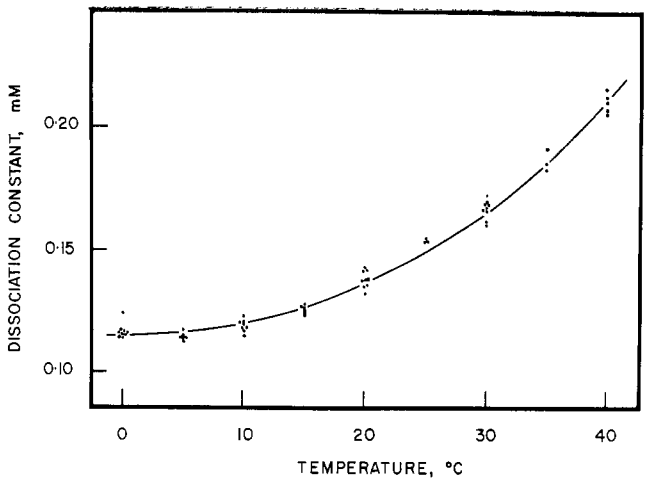

FIGURE 1: Pentane binding to βLGA dimer: the temperature dependence of K_1. Some of the points have been offset horizontally for clarity.

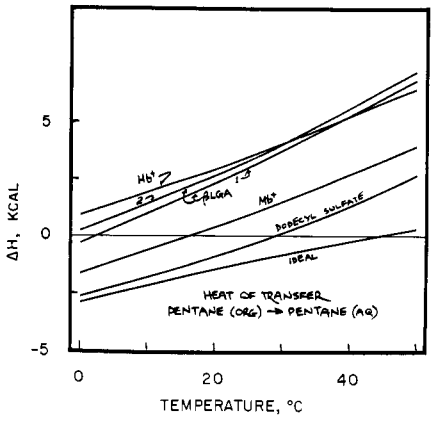

FIGURE 2: The heats of transfer of pentane from nonpolar environments to water.

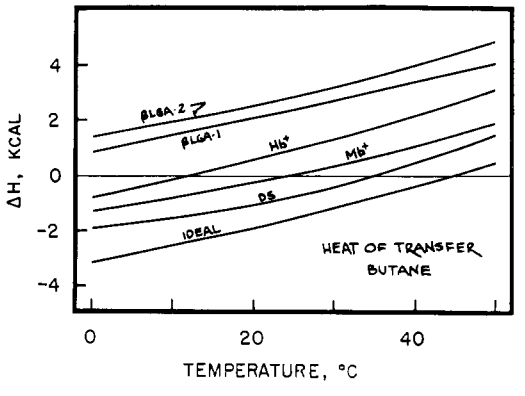

FIGURE 3: The heats of transfer of butane from nonpolar environments to water.

$- 6DT^2$). Whether the data are sufficiently precise to justify this rather than some other temperature dependence for ΔG is an open question and I would not press the point. However, the form, $\Delta G = A + BT + CT \ln T$, which gives a constant

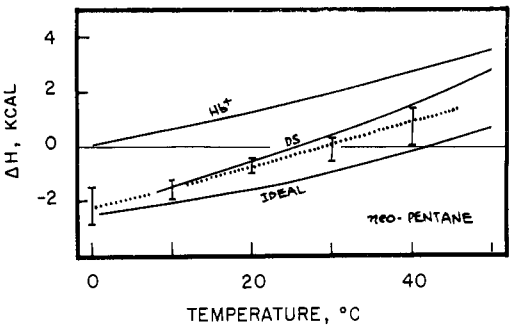

FIGURE 4: The heats of transfer of neopentane from nonpolar environments to water. Dissociation from βLGA-2 is represented by the dotted line (computed for all runs) and error bars (the range of values from several runs) to show that the uncertainty in these data, by far the largest we observe, does not vitiate the conclusions in the text.

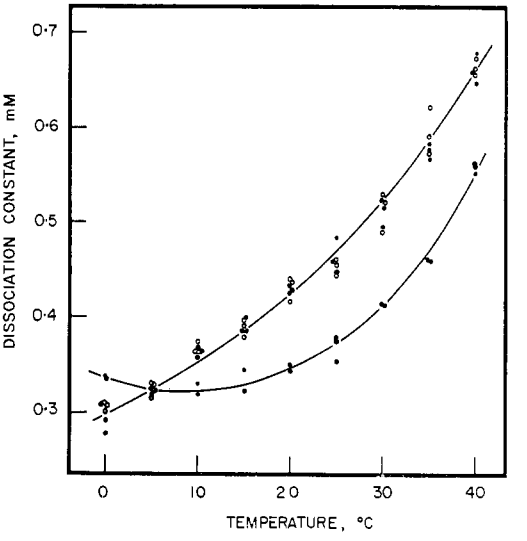

FIGURE 5: Pentane binding to hemoglobins: the temperature dependence of K_1. Open circles, Hb^+CN^-. Filled circles in the (mostly) upper curve, HbO_2; lower curve, Hb. At 5°, the Hb and HbO_2 points coincide—only 3 are shown. The curve calculated for Hb^+-CN^- is within 1% of the HbO_2 curve shown, except at 0°, where it lies 3% higher. The curve for Hb^+, not shown, is intermediate in curvature between HbO_2 and Hb.

ΔC_p, was usually inadequate. The root-mean-square error of ΔG varied from around 15 cal for the strong binding cases to about 60 cal for the weakest binding. ΔH values, as derivatives, are less precise; the good βLG values are probably ±0.2 kcal; and the rest follow. The data for Hb^+, while internally consistent, showed some dependence on the history of the sample, and the accuracy may be ±0.5 kcal at 0° and ±1.0 kcal at 40°.

Results and Discussion

The temperature dependence of K_1 for pentane and βLGA is shown in Figure 1. The temperature dependence of ΔH for dissociation of pentane, butane, and neopentane from all the hydrophobic sites is shown in Figures 2, 3, and 4. All the values of ΔH, ΔS, and ΔC_p at 0 and 25° are given in Table II.

TABLE II: The Thermodynamic Parameters of Alkane Binding (Part a) and Heme Protein Comparison (Part b).[a]

	Pentane			Neopentane			Butane		
	ΔH	ΔS	ΔC_p	ΔH	ΔS	ΔC_p	ΔH	ΔS	ΔC_p
				Part a					
Ideal	−2.9	−33	73	−2.5	−30	33	−3.1	−30	61
	−1.2	−27	64	−1.3	−26	64	−1.5	−25	72
Dodecyl	−2.6	−29	73	−2.4	−27	82	−1.9	−23	27
sulfate	−0.4	−21	103	0	−19	104	−0.7	−19	67
Mb^+	−1.7	−28	92				−1.3	−22	44
	0.9	−19	114				0.1	−18	65
Hb^+	1.0	−20	85	0.1	−19	52	−0.8	−23	64
	3.4	−12	106	1.6	−14	70	1.0	−17	78
βLGA-1	−0.3	−26	119				0.9	−19	61
	3.0	−15	141				2.4	−14	64
βLGA-2	0.2	−25	103	−2.2	−26	72	1.4	−17	47
	3.3	−15	140	−0.3	−19	85	2.9	−12	71
				Part b					
HbO_2	2.3	−16	52						
	3.8	−11	67						
Hb^+CN^-	1.9	−17	62						
	3.7	−11	78						
Hb^+	1.7	−18	99						
	4.5	−8	124						
Hb	−1.6	−30	172						
	3.2	−13	214						
ApoMb	−2.6	−34	200						
	3.1	−14	254						

[a] The reaction is $alkane_{(org)} \rightarrow alkane_{(aq)}$. ΔH is in kilocalories per mole; ΔS and ΔC_p in calories per degree per mole. The upper and lower lines refer to 0 and 25°, respectively.

The data for a pentane series on HbO_2, Hb, Hb^+, and Hb^+CN^- are given in Figure 5. HbO_2 and Hb^+CN^- are almost indistinguishable; Hb^+ somewhat different. Deoxyhemoglobin has a very high ΔC_p, which may argue for some continuous readjustments of the sites or the subunits with temperature. The other example of a very high ΔC_p, if the data, which seem precise, can be taken literally, is apomyoglobin, where anomalous behavior might be expected (interactions at this site are more complicated than in the other cases; Wishnia, 1969).

Except as noted, ΔC_p, for the proteins, and for all solutes, is comparable with the dodecyl sulfate values. This characteristic variation of ΔH with temperature derives from the unusual behavior of water, and is, in fact, diagnostic for "hydrophobic" interactions; in this sense, the hydrophobic sites are well behaved. It is immediately, and strikingly, obvious that the high affinity of β-lactoglobulin and ferrihemoglobin for pentane arises from the large, nearly constant difference (3–4 kcal) between the heat of dissociation of pentane from these proteins and from dodecyl sulfate micelles (see Figure 3). Ferrimyoglobin has an intermediate affinity and an intermediate ΔH. The high affinity for butane has a similar origin. On the other hand, for neopentane, ΔH for β-lactoglobulin is more negative by 1–2 kcal, and ΔH for ferrihemoglobin more positive by 1–2 kcal, than the dodecyl sulfate values. (Mb^+ binds too weakly for accurate measurements.) It is this behavior which accounts for the sharp differentiation between pentane and neopentane by βLG, and the unique instance of stronger binding by Hb^+ than by βLG (Wishnia, 1969).

The results are worth restating: the binding of butane and pentane to Hb^+ and βLG is not only strong, it is anomalously strong; ΔG of dissociation from these proteins is substantially larger than from dodecyl sulfate micelles and even larger than ΔG for the hypothetical transfer from an ideal liquid phase; the larger ΔG arises from a much larger ΔH only partially offset by an increase in ΔS. The origin of the excess ΔH is the key question. (ΔS will be discussed later). It cannot arise from the alkane–water interaction, which is obviously the same for the dodecyl sulfate micelles.

Alkane binding with displacement of water from the binding site, an interesting problem I hope to deal with elsewhere, is precluded for Hb^+ and Mb^+ (Schoenborn *et al.*, 1965, 1967) and very probably for βLG (in any case, the temperature dependence would be wrong); only residue–residue and alkane–residue interactions need be considered. Large-scale rearrangements are ruled out; xenon binding to Mb^+ and Hb^+, cyclopropane binding to Mb^- (Schoenborn *et al.*, 1965, 1967), and iodobutane binding to βLGA (D. W. Green and A. Wishnia, unpublished) at levels of 1 mole/subunit, produce electron density changes only near the site of binding.

Moreover, one cannot get high values of ΔH from exceptionally strong alkane–protein interactions in the complex: we know from solubility studies (*e.g.*, Thomsen and Gjaldbaek, 1963; Kretschmer and Wiebe, 1952) that the van der Waals interaction energy between shorter and longer alkyl chains is maximal (*i.e.*, most negative), and that changes in the excess enthalpy of the solute produced by including aromatic, ether, or alcohol groups in the "solvent" are not only small (0.5 kcal for butane in methanol, an extreme case), but in the opposite direction from what we have to account for. I have argued elsewhere (Wishnia and Pinder, 1964) that exotic interactions can only lead to an overall less favorable ΔG of binding.

We are forced to seek the source of the excess ΔH in some sort of "strain," or locally suboptimal interaction, in the *unoccupied* binding site, and to propose the following mechanism of binding: the thermodynamically stable native protein structure is a compromise in which the binding site gets the short end; if the constraints imposed by the rest of the protein were removed, the site would relax to a new configuration with a decrease in free energy and enthalpy. The site need not, and probably would not look anything like the spatial complement of the "substrate" (an empty pentane-shaped hole is out of the question). However, only molecules of favorable geometry, the *n*-alkanes, can accommodate to the (rearranged) site to provide the interactions equivalent to the hypothetical relaxation, and realize the extra free energy of binding. The site of βLG is so restricted in flexibility and expandability (2 pentane equiv is the upper limit) that neopentane cannot enjoy such a favorable disposition, but must shoulder its way in. This kind of localized "induced fit" may well occur in enzymes, producing unexpectedly high affinities for specific substrates, and strong discrimination against closely related compounds.

The nature of the strain is not known. If the problem is strictly local, as suggested by the limited electron density shifts, then the most straightforward possibility is that the hydrophobic side chains in the site are constrained to occupy a volume larger than the normal volume of a hydrocarbon liquid: the interaction energy of the resulting poor van der Waals contacts is weaker than normal by 3–4 kcal/mole. (The heat of vaporization of pentane is 6.2 kcal/mole.)

Can we get such energies with an abnormal volume large enough to give modest displacements when a molecule of pentane is bound, small enough to be reasonable, and consistent with the observed entropy changes? There is an ambiguity in ΔS arising from the choice of standard states, and a further uncertainty in the choice of ΔS_0 and ΔH_0 for a "normal" site; still, $(\Delta S - \Delta S_0)/(\Delta H - \Delta H_0)$ falls between 1.5 and 2.5 gibbs per kcal. (For comparison, $\Delta S/\Delta H$ of vaporization of pentane at the boiling point is 3.0 gibbs/kcal.) We inquire whether $\Delta S/\Delta H$ is well behaved as we allow the site volume to increase from that of a normal liquid to the point where ΔH = 3–4 kcal. If we use a very crude model, several equivalent side chains in spherical cells, and the rest of the Lennard-Jones and Devonshire apparatus (see eq 49.1–49.15 in the text by Hill, 1956, or Wentorf *et al.*, 1950) we can calculate S and E as functions of the relative volume, V/V^* (at $V/V^* = 1$ the LJD potential is a minimum). Although ΔS and ΔE depend differently upon the choice of V/V^* for the "normal" liquid and on the number, n, of cells into which the site volume is divided, if we keep the energy parameter $z\epsilon/kT$ proportional to $1/n$, choose n = 1, 2, 4, or 8, and choose a normal V/V^* between 1.05 and 1.20 (Wentorf *et al.*, 1950), the range of ΔS and ΔE for a given ΔV is quite narrow. Thus, for a site of four (spherical) "ethanes" ($z\epsilon/kT$ = 40/4, V^* = 200 ml), for ΔV = 80 ml, ΔS = 3.1–4.0 gibbs, ΔE = 2.6–3.0 kcal. Other plausible choices of parameters can raise $\Delta S/\Delta E$ to 2. The crude model is qualitatively consistent with the data.

We can distinguish three kinds of hydrophobic regions. The typical region has good van der Waals contacts (*i.e.*, a normal "free volume" of 0.1–1.0%) but cannot be enlarged to include nonpolar molecules without unfavorable results in the rest of the protein: such sites bind alkanes weakly or not at all (even the hydrophobic sites of βLG and Hb^+, after binding the first molecule of pentane, are examples). Such regions can still be large (*e.g.*, ribonuclease; Wyckoff *et al.*, 1967). A second kind also has good van der Waals contacts, which are preserved after binding, but, like a micelle, is able to enlarge by the molar volume of the included molecule without strain: the enthalpy of binding is therefore like that for dodecyl sulfate micelles (bovine serum albumin is an example; the fact that the region behaves like a solid rather than a liquid, in nuclear magnetic resonance studies, is of no consequence (Saunders and Wishnia, 1958; Bovey *et al.*, 1959; see also Wishnia and Pinder, 1964)). Alkane binding is moderately strong and extensive; we would expect such sites to be large. In the third kind, described above, characterized by a modest superbinding arising from a heat of dissociation 3–4 kcal higher than that of dodecyl sulfate micelles, strain is built into the unoccupied site. This strain may consist in having a larger molar volume (say, by 80 ml), and free volume (5–30 ml), and a less negative interaction energy and entropy, than a normal liquid or solid; the site–solute complex is normal.

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Significance of the Initial Fast Reaction in the Acid Denaturation of Ferrihemoglobins*

John W. Allis and Jacinto Steinhardt

ABSTRACT: Acid denaturation measurements of both horse and human ferrihemoglobins (Hb^+) at 2.5° show that the initial red shift of the Soret band at low pH is slowed to a rate measurable by stop-flow techniques. Parallel changes occur in the visible absorption bands of Hb^+; the bands at 500 mμ and 630 mμ disappear and a new band appears at 525 mμ (horse Hb^+) or 531 mμ (human Hb^+). These changes are suppressed by the presence of formate ion only, among several common anions tried.

These spectral events, which occur only at pH <3, although acid denaturation occurs at much higher pH, are interpreted as changes in the environment of the heme group which affect the iron orbitals coordinating with the ligands. The changes are probably due to the effect of the high-charge density acquired by the protein at low pH values prior to heme separation; contrary to an earlier report, the initial fast reaction is probably *not* important to the over all acid denaturation scheme of Hb^+ previously described; it is a manifestation of an alternative pathway, superimposed on the basic mechanism. In contrast to the result at 25°, the pH–denaturation rate profiles for the two species at low temperature do not approach the same limiting rate; the difference corresponds to the known difference in the final products at high and low temperatures at least with horse ferrihemoglobin.

The acid denaturation of both horse and human ferrihemoglobin (Hb^+), at low ionic strength, followed by changes in their Soret absorption bands, has been previously reported as a three-step sequential process (Polet and Steinhardt, 1969). The first step (A → B) is a very fast reaction (half-life at 25° <10 msec) manifested by a red shift of the Soret band, from 405 to 408 mμ for horse Hb^+ and to 410 mμ for human Hb^+. The second step (B → C) is characterized by the disappearance of the red-shifted Soret band and the simultaneous rise of a new band at 397.5 mμ; it has been shown that this change is caused by the expulsion of the heme from the protein, and the simultaneous or nearly simultaneous collapse of the protein's tertiary structure. The final step (C → D), the disappearance of the band at 397.5 mμ, and the appearance of a broad band at 370 mμ, is caused by the dimerization of the free heme. The three steps are seen as distinct processes at pH values below about 2.4. At higher pH values the second and third steps cannot be separated because the second, the rate of which is pH dependent, becomes rate limiting.

When the acidifying agent is formate buffer instead of HCl, the sequence is essentially the same except that the initial red shift, A → B, is not present (Polet and Steinhardt, 1969). With formate, at protein concentrations high enough so that the second-order reaction C → D is so fast that the rate measured is that of the first-order reaction B → C alone, identical kinetic results have been obtained at the Soret band and at 630 mμ (Zaiser and Steinhardt, 1953).

In this paper the original intent was to extend the earlier spectroscopic studies to temperatures as low as 2.5° and to pH ranges down to 1.8 in order to examine the kinetics of the initial very fast reaction (A → B) and to carry out measurements in the visible (490–670 mμ) as well as in the Soret region (350–425 mμ). It became immediately clear that the first very rapid reaction (A → B) is time dependent rather than instantaneous, and that drastic changes in the visible spectral region occur which can be correlated with coincident changes of the Soret spectrum. The results are reported here, together with interpretations which throw light on the interaction of heme and globin and on the stabilization of hemoglobin by its heme linkages.

* From the Department of Chemistry, Georgetown University, Washington, D. C. 20007. *Received September 10, 1969.* Supported by Grants GB 6848 (National Science Foundation) and HE 12256-05A2 (National Institutes of Health).