

tion, followed by *chromatography* in the perpendicular direction. Finally, the peptide spots were made visible by staining the filter paper with ninhydrin. This sequence of steps—selective cleavage of a protein into small peptides, followed by their separation in two dimensions—is called *fingerprinting*.

The fingerprints of hemoglobins A and S were highly revealing (Figure 7-46). When they were compared, *all but one of the peptide spots matched*. The spot that differed was eluted from each fingerprint and shown to be a single peptide consisting of eight amino acids. Amino acid analysis indicated that this peptide in hemoglobin S differed from the one in hemoglobin A by a single amino acid. Ingram determined the sequence of this peptide and showed that *hemoglobin S contains valine instead of glutamate at position 6 of the β chain*:

Hemoglobin A	Val-His-Leu-Thr-Pro	Glu-Glu-Lys
Hemoglobin S	Val-His-Leu-Thr-Pro	Val-Glu-Lys
	$\beta 1 \quad 2 \quad 3 \quad 4 \quad 5 \quad 6 \quad 7 \quad 8$	

STICKY PATCHES ON DEOXYGENATED HEMOGLOBIN S LEAD TO THE FORMATION OF FIBROUS PRECIPITATES

The substitution of valine for glutamate at position 6 of the β chains places a nonpolar residue on the outside of hemoglobin S (Figure 7-47). The oxygen affinity and allosteric properties of hemoglobin are virtually

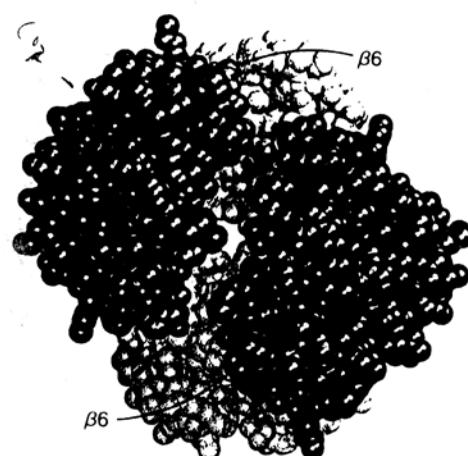


Figure 7-47
The positions of the amino acid changes (glutamate to valine at residue 6 of each β chain) are shown in red in this model of deoxyhemoglobin. Note that $\beta 6$ is located at the surface of the protein. The α chains are shown in yellow, and the β chains in blue. [Drawn from 4hhb.pdb. G. Fermi, M.F. Perutz, B. Shaanan, and R. Fourme. *J. Mol. Biol.* 175(1984):159.]

unaffected by this change. However, this alteration markedly reduces the solubility of the deoxygenated but not the oxygenated form of hemoglobin. The reason is that the valine side chain of hemoglobin S interacts with a complementary sticky patch on another hemoglobin molecule (Figure 7-48). The complementary site, formed by phenylalanine $\beta 85$ and leucine $\beta 88$, is exposed in deoxygenated but not in oxygenated hemoglobin. Thus, sickling occurs when there is a high concentration of the deoxygenated form of hemoglobin S.

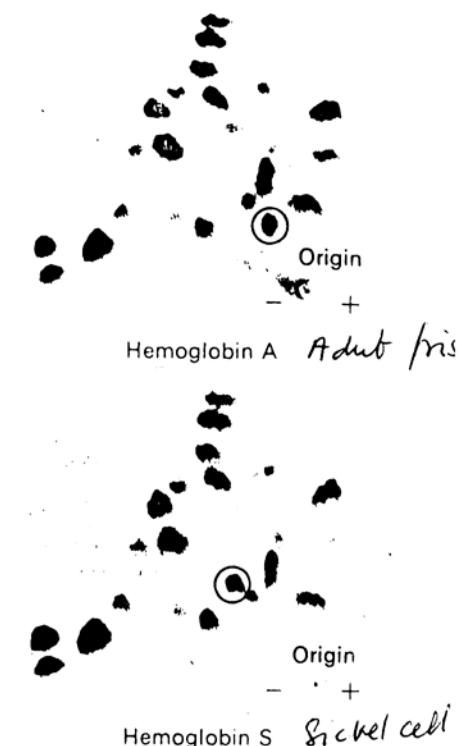
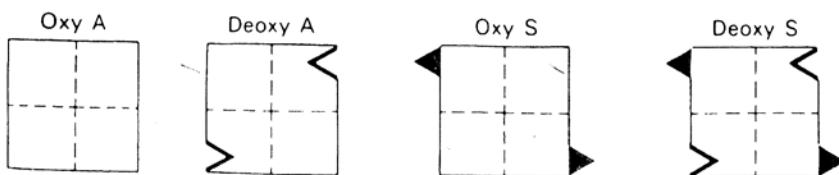


Figure 7-46
Comparison of the ninhydrin-stained fingerprints of hemoglobin A and hemoglobin S. The position of the peptide that is different in these hemoglobins is encircled in red. [Courtesy of Dr. Corrado Baglioni.]

Solubiliteten är bättre
då Häm. A är närvana

Figure 7-48
The red triangle represents the sticky patch that is present on both oxy- and deoxyhemoglobin S but not on either form of hemoglobin A. The complementary site is represented by an indentation that can accommodate the triangle. This complementary site is present in deoxyhemoglobin S and is probably also present in deoxyhemoglobin A.

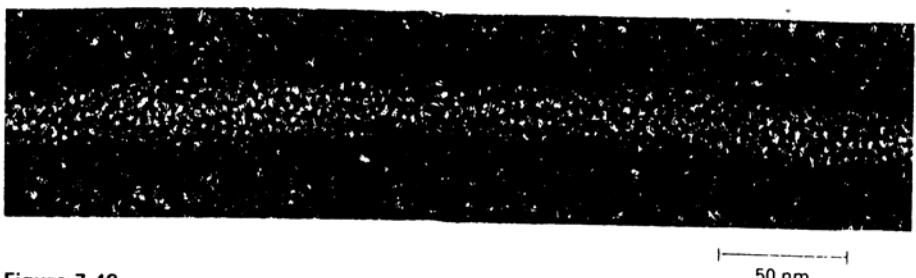


Figure 7-49

Electron micrograph of a negatively stained fiber of deoxyhemoglobin S. [From G. Dykes, R.H. Crepeau, and S.J. Edelstein. *Nature* 272(1978):509.]

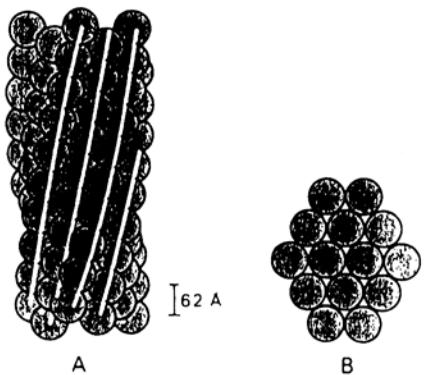


Figure 7-50
Fourteen-stranded helical model of the deoxyhemoglobin S fiber: (A) axial view; (B) cross-sectional view. Each circle represents a hemoglobin S molecule. [After G. Dykes, R.H. Crepeau, and S.J. Edelstein. *Nature* 272(1978):509.]

Further insight into sickle-cell disease comes from examining the nature of the precipitate formed by deoxygenated hemoglobin S, which deforms the red cell. Electron microscopy shows that the precipitate consists of fibers having a diameter of 21.5 nm (Figure 7-49). Each fiber is a fourteen-stranded helix made of seven strongly interacting pairs of hemoglobin molecules (Figure 7-50). Multiple polar interactions, in addition to the critical one between sticky patches, stabilize the fiber.

What determines whether a red cell becomes sickled during its passage through the capillary circulation, which takes about a second? The striking experimental finding is that the rate of fiber formation is proportional to about the tenth power of the effective concentration of deoxyhemoglobin S. Thus, fiber formation is a highly concerted reaction. These kinetic data indicate that nucleation is the rate-limiting phase in fiber formation. The fiber grows rapidly once a critical cluster of about 10 deoxyhemoglobin S molecules has been formed. The important clinical implication is that *kinetic*, as well as thermodynamic, factors are important in sickling. A red cell that is supersaturated with deoxyhemoglobin S will not sickle if the lag time for fiber formation is longer than the transit time from the peripheral capillaries to the alveoli of the lungs, where reoxygenation occurs.

These facts account for several clinical characteristics of sickle-cell anemia. A vicious cycle is set up when sickling occurs in a small blood vessel. The blockage of the vessel creates a local region of low oxygen concentration. Hence, more hemoglobin goes into the deoxy form and so more sickling occurs. The very strong dependence of the polymerization rate on the concentration of deoxyhemoglobin S also accounts for the fact that people with sickle-cell trait are usually asymptomatic. The concentration of deoxyhemoglobin S in the red cells of these heterozygotes is about half that in homozygotes, and so their rate of fiber formation is about a thousandfold slower (30 s, compared with 30 msec).

THE HIGH INCIDENCE OF THE SICKLE GENE IS DUE TO THE PROTECTION CONFERRED AGAINST MALARIA

The frequency of the sickle gene is as high as 40% in certain parts of Africa. Until recently, most homozygotes have died before reaching adulthood, and so there must have been strong selective pressure to maintain the high incidence of the gene. James Neel proposed that the heterozygote enjoys advantages not shared by either the normal homozygote or the sickle-cell homozygote. Indeed, sickle-cell trait confers a small but highly significant degree of protection against the most lethal form of malaria, perhaps by accelerating the destruction of infected erythrocytes. In a malaria-infested region, the reproductive fitness of a person with sickle-cell trait is about 15% higher than that of someone with normal hemoglobin. The incidence of malaria and the frequency of the sickle

Lysozyme

A search was then made for possible catalytic groups close to the glycosidic bond that is cleaved. A *catalytic group* is one that directly participates in making or breaking covalent bonds. The donation or abstraction of a hydrogen ion is a critical step in most enzymatic reactions. Hence, the most likely candidates are groups that can serve as *proton donors or acceptors*. The only plausible catalytic residues near the glycosidic bond that is cleaved by lysozyme are aspartic acid 52 and glutamic acid 35. The aspartic acid residue is on one side of the glycosidic linkage, and the glutamic acid residue is on the other. These two acidic side chains have markedly different environments. Aspartic acid 52 is in a distinctly polar environment, where it serves as a hydrogen bond acceptor in a complex network of hydrogen bonds. In contrast, glutamic acid 35 lies in a nonpolar region. Dissociation of a proton from a carboxyl group is less favored in a nonpolar than in a polar environment. Hence, at pH 5, the pH optimum for the hydrolysis of chitin by lysozyme, *aspartic acid 52 is probably in the ionized COO⁻ form (aspartate), whereas glutamic acid 35 is in the un-ionized COOH form*. The nearest oxygen atom of each of these acid groups is located about 3 Å away from the glycosidic linkage (Figure 9-10).

A CARBONIUM ION INTERMEDIATE IS CRITICAL FOR CATALYSIS

Phillips and his colleagues proposed a detailed catalytic mechanism for lysozyme based on the preceding structural data. The essential steps are

1. The -COOH group of glutamic acid 35 donates an H⁺ to the glycosidic oxygen atom between rings D and E. The transfer of a proton cleaves the bond between C-1 of the D ring and the glycosidic oxygen atom (Figure 9-11).
2. This creates a positive charge on C-1 of the D ring. This transient species is called a *carbonium ion* because it contains a positively charged carbon atom. It is also known as an *oxocarbonium ion* because some of the positive charge is shared by the ring oxygen atom.
3. The dimer of NAG consisting of residues E and F diffuses away from the enzyme.

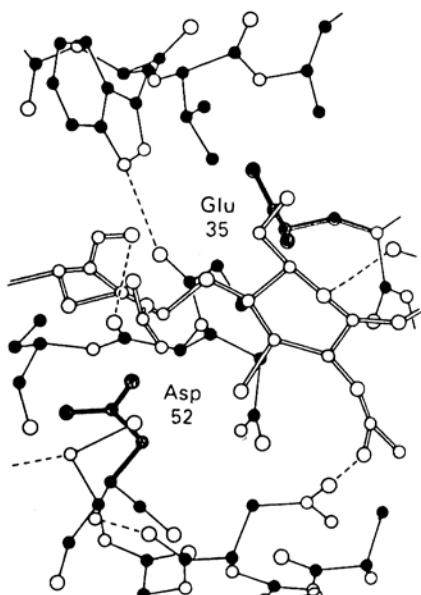


Figure 9-10
Structure of part of the active site of lysozyme. The D (left) and E (right) rings of the hexa-NAG substrate are shown in yellow. The side chains of aspartate 52 (red) and glutamic acid 35 (green) are in close proximity.
[After W.N. Lipscomb. Proc. Robert A. Welch Found. Conf. Chem. Res. 15(1971):150.]

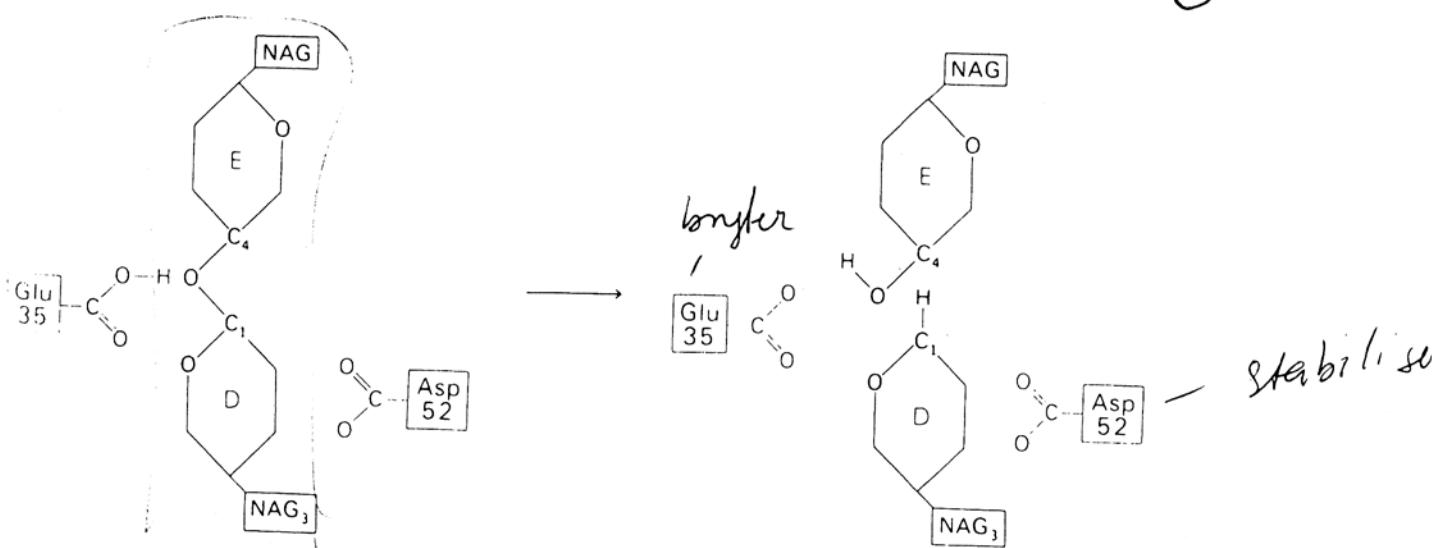


Figure 9-11

The first step in catalysis by lysozyme is the transfer of an H⁺ from Glu 35 to the oxygen atom of the glycosidic bond. The glycosidic bond is thereby cleaved, and a carbonium ion intermediate is formed.

4. The carbonium ion intermediate then reacts with OH^- (or H_2) from the solvent (Figure 9-12). Glutamic acid 35 becomes reprotonal and tetra-NAG, consisting of residues A, B, C, and D, diffuses away from the enzyme. Lysozyme is then ready for another round of catalysis.

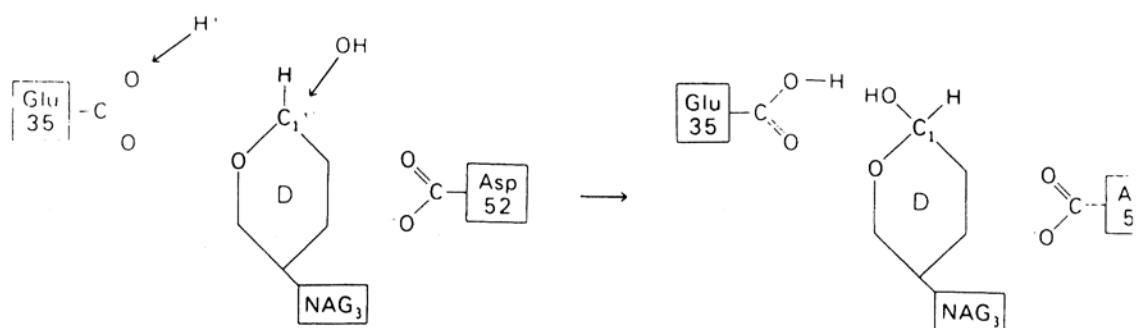


Figure 9-12

The cleavage reaction is completed by the addition of OH^- to the carbonium ion intermediate and H^+ to the side chain of Glu 35.

The critical elements of this proposed catalytic scheme are

1. *General acid catalysis.* A proton is transferred from glutamic acid 5 which is un-ionized and optimally located 3 Å away from the glycosidic oxygen atom. The term *general acid* indicates that the source of the donor group rather than free H^+ .

2. *Promotion of the formation of the carbonium ion intermediate.* The enzymatic reaction is markedly facilitated by two different factors that stabilize the carbonium ion intermediate:

a. The *electrostatic factor* is the presence of a negatively charged group 3 Å away from the carbonium ion intermediate. Aspartate 5 which is in the negatively charged carboxylate form, electrostatically stabilizes the positive charge on C-1 of ring D.

b. The *geometrical factor* is the distortion of ring D (Figure 9-13). Hexa-NAG fits best into the active-site cleft if sugar residue D is distorted out of its customary chair conformation into a half-chair form. This distortion enhances catalysis because the half-chair geometry markedly promotes the formation of the carbonium ion. In the half-chair form, the planarity of carbon atoms 1, 2, and 5 and the ring oxygen atom enables the positive charge to be shared by resonance between C-1 and the ring oxygen atom.

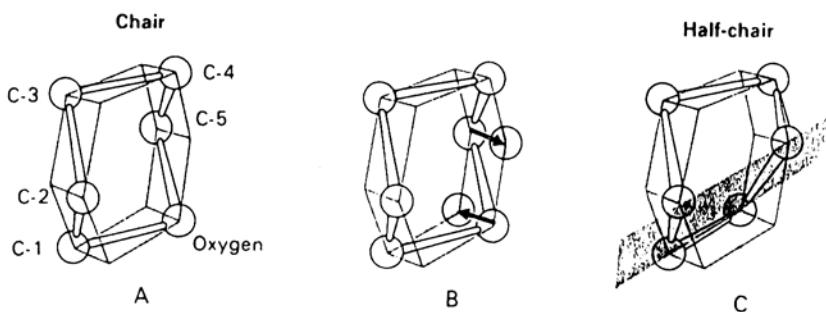


Figure 9-13

Distortion of the D ring of the substrate of lysozyme into a half-chair form: (A) a sugar residue in the normal chair form; (B) on binding to lysozyme, the ring oxygen atom and C-5 of sugar residue D move so that C-1, C-2, C-5, and O are in the same plane, as shown in part C. [After D.C. Phillips. The three-dimensional structure of an enzyme molecule. Copyright © 1966 by Scientific American, Inc. All rights reserved.]