

INTRAMOLECULARLY CROSS-LINKED HEMOGLOBIN AND METHOD OF PREPARATION

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FIELD OF THE INVENTION

The present invention relates to hemoglobin which has been intramolecularly cross-linked with reagents which transform hemoglobin into a physiologically competent oxygen carrier. As defined herein, "physiologically competent" or "physiologically acceptable" with respect to the oxygen carrier means that the oxygen carrier can absorb oxygen at the partial pressures of oxygen prevailing at the site of oxygenation of hemoglobin, e.g., in the lungs of men or other air-breathing organisms and in the gills of fish, and release it to the tissues of the same organisms in amounts which are life supporting at least when the organisms are in a resting state.

BACKGROUND OF THE INVENTION

1. Stroma-Free Hemoglobin

Intravenously injected (infused) crude hemolysates and extensive hemolytic processes produced in vivo by immunological reactions involving intravascular lysis of red blood cells, are known to produce a clinical syndrome characterized by disseminate intravascular coagulation. This syndrome is often fatal and is produced by the residual red blood cell walls (stroma) and their fragments, so infused into circulating blood. Stroma-free hemolysates do not show this toxicity (See Rabiner, S. F. et al, *J. Exp. Med.*, 126:1127 (1967)). As a result, it has been desired to use stroma-free hemoglobin as an oxygen carrier in cell-free transfusional fluids.

However, the use of stroma-free hemoglobin has the following two disadvantages. In vivo, the retention time of the stroma-free human hemoglobin is very short, i.e., it has a half-life on the order of 1-4 hours (See Rabiner, S. F., supra, and De Venuto, F. et al, *Transfusion*, 17:555 (1977)). "Half-life" is defined as the time necessary to eliminate 50% of the infused hemoglobin from circulating blood. Further, outside of the red blood cells, hemoglobin has a high affinity for oxygen which, in vivo, would prevent the release, i.e., the transport, of oxygen from hemoglobin to the tissues. These disadvantages are directly the result of the molecular structure of hemoglobin.

Hemoglobin is a tetrameric molecule having a molecular weight of 64,500 Daltons. The tetrameric molecule is formed of two pairs of alpha and beta subunits. The subunits are held together as a result of ionic and Van der Waals forces, and not as a result of covalent bonds. When hemoglobin is oxygenated, i.e., combined with oxygen, it readily forms alpha-beta dimers having a molecular weight of 32,250 Daltons. These dimers are not retained in vivo by the kidneys and are eliminated through the urine.

The tetrameric structure of hemoglobin also provides a binding site for 2,3-diphosphoglycerate. Inside red blood cells, 2,3-diphosphoglycerate combines with hemoglobin in order to decrease its oxygen affinity to a level compatible with oxygen transport. The binding of 2,3-diphosphoglycerate and hemoglobin is purely electrostatic and no stable covalent bonds are formed. Thus,

when red blood cells are ruptured and 2,3-diphosphoglycerate is not retained inside the cells by the cell wall, it is released from hemoglobin. As a result, hemoglobin acquires a higher oxygen affinity. This prevents the transport of oxygen from hemoglobin to the tissues. The level of this higher affinity is sufficient such that the oxygen affinity can be considered "non-physiological".

Because of the many appealing qualities of hemoglobin i.e., its ability to reversibly bind oxygen, the low viscosity of a hemoglobin solution and its easy preparation and storage for long periods of time, various attempts have been made in order to overcome the above-described disadvantageous characteristics of stroma-free hemoglobin. These various attempts are discussed in more detail below.

2. Chemical Treatments for Preventing the Formation of Dimers

The formation of alpha-beta dimers, which are not retained in vivo, can be prevented by coupling the tetrameric molecules of hemoglobin with large molecular weight matrices, ranging from 20,000 to 275,000 Daltons, for example, matrices such as dextran (See Tam, S. C. et al, *Can. J. Biochem.*, 56:981 (1978) and Bonneaux, F. et al, *Experientia*, 37:884 (1981)) and hydroxyethyl starch (See Baldwin, J. E. et al, *Tetrahedron*, 37:1723 (1981)). This coupling prevents the elimination of hemoglobin in vivo from the kidneys by way of the urine. Other types of polymeric coupling employing collagen, collagen degradation products, and gelatin as a supporting matrix have also been employed (See U.S. Pat. No. 2,591,133, U.S. Pat. No. 3,057,782, and Bowes, F. et al, *Biochem. Biophys. Acta*, 168:341 (1968)). However, the oxygen affinity of the resulting coupled hemoglobin is even higher than that of stroma-free hemoglobin and thus hemoglobin coupled in this manner cannot be advantageously employed as an oxygen transport medium.

Other known treatments for preventing the formation of alpha-beta dimers are based on reactions which polymerize the tetrameric molecules of hemoglobin to form so-called "polyhemoglobins". Polyhemoglobins can be obtained using bifunctional reagents such as glutaraldehyde (See Hopwood, C. et al, *Histochem. J.*, 2:137 (1970) or diimidate esters (See Mock, W. et al, *Fed. Proc.*, 34:1458 (1975)) and U.S. Pat. No. 3,925,344). These bifunctional reagents form covalent bonds between the amino groups present on the surface of different hemoglobin molecules producing intermolecular cross-links. There are 40 or more of such amino groups belonging to lysyl residues on the surface of mammalian hemoglobins (44 in human hemoglobin) so that a large number of possible combinations of hemoglobin molecules occur. As a result, the polyhemoglobin reaction products are a heterogeneous mixture of various molecular species which differ in size and shape. The molecular weights thereof range from 64,500 to 600,000 Daltons. The separation of individual molecular species from the heterogeneous mixture is virtually impossible. In addition, although longer retention times in vivo are obtained using polyhemoglobins, the oxygen affinity thereof is higher than that of stroma-free hemoglobin.

Besides the various treatments discussed above which result in formation of heterogeneous mixtures of polyhemoglobin, reagents have been developed which are capable of producing an internal cross-link of the hemoglobin subunits with little or no formation of polyhemoglobins. More specifically, the formation of cross-links between the beta subunits of hemoglobin