The Amino Acid Composition of the Haemoglobins of the Domestic Fowl

By R. L. ALLEN* and W. N. M. RAMSAY. (*Department of Biochemistry, University of Edinburgh Medical School, Teviot Place, Edinburgh 8, U.K.*)

Previous analyses (van der Helm & Huisman, 1958; Saha, 1964) of the amino acid composition of the haemoglobins (Hb I and Hb II) of the domestic fowl disagree, particularly in the values for the basic and acidic amino acids. Neither paper offers evidence of the purity of the fractions analysed.

In the present work the haemoglobins have been separated on CM-cellulose by a technique derived from that of Matsuda & Takei (1963), who adsorbed the haemoglobins from a erythrocyte lysate in phosphate buffer (10mM, pH 6.9) and eluted Hb II and Hb I in that order by a buffer concentration gradient at the same pH. In our hands Hb II was not initially adsorbed to the column unless the pH was lowered to 6.3. Elution by a buffer concentration gradient at pH 6.3 separated the haemoglobins, but a sensitive new criterion, based on the ratios of extinctions at selected wavelengths over the whole of each elution peak, disclosed contamination of Hb II, possibly by a non-haem protein. The contaminant could be removed by washing the column with phosphate buffer (10mM, pH 6.9) before elution of the haemoglobins either by concentration gradient or stepwise (Hb II, 40mM; Hb I, 100mM). Both haemoglobins then appeared pure by electrophoresis on polyacrylamide gel as well by the new test.

The globins were prepared from desalted haemoglobin solutions by acetone-HCl precipitation and hydrolysed in 6M-HCl for 24h at 105°C. Amino acid analyses were done by the method of Spackman, Moore & Stein (1958) and the amino acid proportions calculated as the best integers to fit a molecular weight of about 62000 for each globin.

The molecular weights used by the earlier workers were higher and show some unexplained variations, but after allowance is made for such discrepancies our results agree quite closely with those of Saha (1964). Van der Helm & Huisman (1958) found a marked excess of basic over acidic amino acids in Hb I and the converse in Hb II, and the more modest difference found in our work, particularly when the amide nitrogen is taken into account, agrees better with the observed electrophoretic and ion-exchange properties of the two proteins.

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* Present address: Department of Botany, University of Hull, Hull HU6 7RX, U.K.

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Antibodies of Restricted Heterogeneity to an Antigenic Determinant of Sperm-Whale Myoglobin

By R. J. BOEGMAN and M. J. CRUMPTON. (*National Institute for Medical Research, London N.W.7, U.K.*)

Antibodies produced in response to immunization with natural and artificial antigens are invariably heterogeneous. It has been suggested that this heterogeneity reflects, at least partly, the complexity of the antigenic structure (Singer, 1964). This suggestion is supported by the restricted heterogeneity of the antibodies formed to some polysaccharides (Braun & Krause, 1968), and to structurally homogeneous hapten-protein or hapten-polypeptide conjugates (Brenneman & Singer, 1968; Richards, Pincus, Bloch, Barnes & Haber, 1969). In this case, antibodies of restricted heterogeneity should also be formed against each of the antigenic determinants of a globular protein; each determinant is believed to correspond to a unique and restricted portion of the surface of the protein molecule (Crumpton, 1968). Indeed, some evidence has been obtained that the antibodies present in anti-(sperm-whale myoglobin) sera to the C-terminal heptapeptide of myoglobin represent a restricted population (Givas, Centeno, Manning & Sehon, 1968). In the present investigation the degree of heterogeneity of the antibodies in anti-(sperm-whale myoglobin) sera to the C-terminal 22-amino acid portion of myoglobin was determined by isoelectric focusing in polyacrylamide gel. The C-terminal 22-amino acid peptide of myoglobin has been shown previously to contain at least one, and possibly no more than one, of the antigenic determinants of myoglobin (Crumpton, 1967).

Antibodies to the C-terminal 22-amino acid portion of myoglobin present in antisera obtained from individual rabbits immunized with metmyoglobin in Freund's adjuvant (Crumpton & Wilkinson, 1965) were isolated by using two procedures. First, antibodies were adsorbed to a column of metmyoglobin coupled to activated Sepharose 4B (Axén, Porath & Ernback, 1967) and the column was eluted with a solution of the C-terminal 22-amino acid peptide. Secondly, the antibody fraction of anti-metmyoglobin sera that was adsorbed by the C-terminal 22-amino acid peptide coupled at pH 6.5 to activated Sepharose 4B (Cuatrecasas, 1969), was eluted with m-propionic acid, pH 2.5.

The eluted proteins combined with but did not precipitate ^{125}I -labelled metmyoglobin. Examination of the eluted antibodies by isoelectric focusing (pH 3–10) in 5% (w/v) polyacrylamide gel (Awdeh, Williamson & Askonas, 1968) revealed a small number of bands; antibodies isolated from different antisera gave similar patterns. By contrast, antibodies to whole myoglobin obtained from the metmyoglobin–Sephadex immunosorbent by elution with *m*-propionic acid gave a very much more complex pattern. The results will be discussed in relation to the structure of the C-terminal antigenic determinant of myoglobin.

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Methine-Bridge Specificity of the Coupled Oxidation of Myoglobin and Haemoglobin with Ascorbate

By P. Ó CARRA and EMER COLLERAN. (*Department of Biochemistry, University College, Galway, Irish Republic*)

Ascorbate-coupled oxidation of haem as the pyridine haemochrome occurs randomly at all four methine bridges, ultimately yielding four isomeric biliverdins (Rüdiger, 1968; Ó Carra & Colleran, 1969a), but similar oxidation of haem in myoglobin is specific for the α -methine bridge (Ó Carra & Colleran, 1969a,b).

Applying previously described analytical techniques (Ó Carra & Colleran, 1969a, 1970; Rüdiger, 1968) we find that when the protohaem moiety of myoglobin is replaced by mesohaem or deuthaem (Smith & Gibson, 1959) the specificity of the α -methine-directed oxidation is largely retained, confirming that the directional effect is attributable to the influence of the protein environment around the haem group.

When coupled oxidation of pyridine haemochrome was carried out in hydrophobic solvents

(e.g. benzene–butan-2-one, 1:1, v/v) rather than in the usual aqueous mixtures, the initial step, hydroxylation of a methine bridge, was considerably accelerated but still random. Such an accelerating effect might account for the specificity for the α -methine bridge in myoglobin since the environment around this bridge seems to be particularly hydrophobic.

Haemoglobin differs in a noteworthy way from myoglobin in directing oxidation partly to the α -methine bridge and partly to the β -bridge (Ó Carra & Colleran, 1969a). The proportion of β -methine oxidation varies with species and is particularly high (about 40%) for human haemoglobin.

Incubation of human haemoglobin with *p*-hydroxymercuribenzoate, which causes its dissociation into monomeric subunits (Bucci & Fronticelli, 1965), did not eliminate the β -methine oxidation. The individual α and β subunits (separated as described by Geraci, Parkhurst & Gibson, 1969) both retained the dual specificity, although the proportion of β -methine oxidation was decreased in both, and was particularly low in the isolated β subunit. The difference in specificity between myoglobin and haemoglobin is not therefore primarily attributable to interchain interactions. Hydrophobic environments might be invoked again, however, as a possible explanation, since the environment around the β -methine bridge in both the α and β subunits appears, in the molecular models of haemoglobin (cf. Perutz, 1969), to be more hydrophobic than in myoglobin, though not as hydrophobic as the environment of the α -methine bridge.

Diphosphoglycerate, which allosterically changes the oxygen-binding characteristics of haemoglobin (cf. Benesch & Benesch, 1969) did not change the specificity of the coupled oxidation significantly, but complexing with haptoglobin (Connell & Smithies, 1959) caused a considerable decrease in the proportion of β -methine oxidation, a fact with possible implications in studies of haemoglobin catabolism (cf. Ó Carra & Colleran, 1969a).

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