Imidazole Groups of Iron-Saturated Transferrin

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Electron-paramagnetic-resonance studies have indicated that two nitrogen atoms are co-ordinated with each iron atom in the iron–transferrin complex (Windle, Wiersema, Clark & Feeney, 1963). Subsequent work from this laboratory has suggested that the nitrogen atoms involved were contributed by the imidazole residues (Line, Grohlich & Bezkorovainy, 1967). In addition to the histidyl residues, each iron atom of transferrin is believed to be co-ordinated with three tyrosyl groups and one HCO_3^- ion (Windle *et al.* 1963; Line *et al.* 1967; Tan & Woodworth, 1969). The most promising approach toward the elucidation of the structure of the iron-binding sites of transferrin appears to be the method of differential labelling (Singer, 1967). Our laboratory has established that both the tyrosyl and the amino groups could be expected to serve this function, because a number of such groups are protected by iron against chemical modification (Line *et al.* 1967; Bezkorovainy, Zschocke & Grohlich, 1969). The present communication reports the results of an investigation on the possible use of the histidyl residues for the purpose of differential labelling of the iron-binding sites of transferrin. The histidyl groups are of special interest in this regard, because they appear to be directly involved in the chelation of iron by transferrin.

Materials and methods. Human serum transferrin (iron-free) was the product of Behringwerke A.G. (Marburg/Lahn, Germany), and its molecular weight was assumed to be 83 000. Iron-saturated transferrin was prepared by incubating this preparation with 4 mM-ferrie chloride–citrate complex in 0.1 M-sodium hydrogen carbonate in a ratio of two iron atoms per transferrin molecule. The incubation was continued for 24 h at room temperature, after which the preparation was dialysed and freeze-dried. Bromoacetic acid was obtained from K & K Laboratories (Plainview, N.Y., U.S.A.). All other reagents came from Fisher Scientific Co. (Chicago, Ill., U.S.A.) and were of reagent-grade quality.

The carboxymethylation reaction was carried out generally as described by Line *et al.* (1967), except that 0.4 M-bromoacetic acid was used as the alkylating agent and sodium cacodylate buffer was used as the solvent. In a typical experiment 200 mg of iron-saturated transferrin was dissolved in 5 ml of 0.5 M-sodium cacodylate buffer, pH 6.9, and then

2 ml of 2 M-bromoacetic acid solution previously adjusted to pH 6.9 with 5 M-sodium hydroxide was added. The reaction mixture was immediately diluted to 10 ml with the cacodylate buffer and was left at room temperature in the dark for up to 15 days. Samples (1–2 ml) were removed from the reaction mixture at various time-intervals and were immediately and vigorously dialysed against large volumes of water and then freeze-dried. The pH of the reaction mixture was checked at the time of sampling and was adjusted to pH 6.9 with 5 M-sodium hydroxide whenever necessary. An appropriate control was maintained in the presence of 0.4 M-sodium acetate.

The determination of iron-binding properties of transferrin preparations was based on the property of the iron-transferrin complex to absorb at 465 nm. The specific methodology used was described previously (Bezkorovainy & Grohlich, 1970), whereby the iron-binding activity of transferrin was taken to be 100% at an E_{465}/E_{280} ratio of 0.050. The extent of protein modification was evaluated by the automatic amino acid analyser method by using the system of Moore, Spackman & Stein (1958). The number of histidyl groups modified was evaluated from the difference between the histidine contents of the native and the carboxymethylated samples. Proteins were generally hydrolysed after the removal of residual iron as previously described (Bezkorovainy & Grohlich, 1970). Protein concentrations were determined in each sample spectrophotometrically by using the Zeiss PMQ II spectrophotometer. Optical rotations, ultracentrifugations and moving-boundary-electrophoretic analyses were also done as previously described (Bezkorovainy & Grohlich, 1970).

Results. All carboxymethylated transferrin samples showed single boundaries in the ultracentrifuge and by moving-boundary-electrophoresis methods. The carboxymethylation reaction was relatively rapid in the first 48 h, with some 11–12 histidyl residues being modified during that period of time. The numbers of histidyl groups thus modified were identical for both the iron-saturated and the iron-free transferrin samples. An additional reaction time of 12 days under the same conditions resulted in the modification of only two to four additional histidyl residues. The maximum number of histidyl groups modified was only 14 out of the 23 present in transferrin.

Fig. 1. Effect of 0.4M-bromoacetic acid on the iron-binding properties of transferrins: ○, iron-saturated transferrin control; ●, iron-saturated transferrin; ×, iron-saturated transferrin with residual iron removed and reconstituted with the respective samples; □, iron-free transferrin with iron added after each sampling.

The iron-binding properties of modified transferrins are given in Fig. 1, which shows that the iron-saturated transferrin lost its ability to absorb at 465nm as a result of the carboxymethylation procedure, whereas the control retained its colour even after 14 days reaction time. The iron-free transferrin, included for comparison purposes, also lost its ability to bind iron. When residual iron was removed from the modified iron-saturated transferrin samples and then reconstituted with the same respective samples, the amount of iron bound was identical with that originally present.

Ultracentrifugal analyses revealed that the modified transferrin with 14 histidyl groups carboxymethylated had a sedimentation coefficient of 4.6S at a concentration of 0.5%. Native transferrin had a sedimentation coefficient of 4.8S at the same concentration. Specific rotations of the modified transferrin were -51° , -53° , -62° , -111° and -189° at 589, 578, 546, 436 and 365nm respec-

tively. The respective values for native transferrin were -47° , -49° , -56° , -100° and -176° . The above physical parameters of modified transferrin were performed after the removal of its residual iron.

Discussion. The work presented in the present paper indicates that iron does not protect the imidazole groups of transferrin against carboxymethylation. In fact, such modification of iron-saturated transferrin resulted in a loss of its iron. This finding supports our previous suggestion to the effect that the imidazole groups are involved in chelation of iron by transferrin, but precludes the possibility of using the histidyl groups for the differential labelling of the iron-binding sites of transferrin. Physical studies on the modified transferrin indicated that the carboxymethylation did not result in an extensive alteration of the secondary and tertiary structure of the protein; hence the loss of iron can be ascribed to a *bona fide* modification of the iron-binding ligands of transferrin. This work also shows that some 11–12 histidyl groups of transferrin are very reactive towards bromoacetic acid, whereas the other 10–11 groups are unreactive and may be considered to be in a partially buried state.

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