Biosynthesis of rabbit haptoglobin: Chemical evidence for a single chain precursor

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The primary translation product of the mRNA for rabbit haptoglobin was obtained from a rabbit reticulocyte lysate cell-free system by immunoprecipitation with an antiserum that was directed to the β chain of haptoglobin. Analysis of the translation product by gel electrophoresis and by protein sequencing analysis identified a single polypeptide of M_r 41000. Sequence analysis established a signal region of 18 residues that was immediately followed by the α chain sequence. These results give strong evidence that haptoglobin is initially synthesized as a single chain composed of a signal peptide followed by α and β chain regions, respectively.

Haptoglobin *Cell-free translation* *Signal sequence* *Precursor protein*

1. INTRODUCTION

Human Hp 1-1 is a plasma α_2-glycoprotein that is composed of two dissimilar chains which are covalently arranged in a tetrachain structure, $\alpha_2^1\beta_2$. The primary structure and covalent association of the chains of human Hp 1-1 were previously reported [1]. There are two additional human phenotypes, Hp 2-2 and Hp 2-1, which occur in plasma as a polymeric series [2]. Rabbit [3,4] and most other animal species of haptoglobin with the exception of bovine [5] and canine [6], resemble human Hp 1-1 in regard to tetrachain structure.

We have previously presented strong evidence that Hp is a homologue of the chymotrypsinogen family of serine proteases [1]. The chemical similarity of Hp 1-1 to the serine proteases and the fact that the serine proteases are typically synthesized as single chain precursors led us to propose [1,7] that Hp may also be synthesized as a single chain which is then subsequently hydrolyzed by limited proteolysis. Further evidence for a single chain precursor was provided by biosynthetic studies of rabbit Hp [8], in which a single translation product of M_r 43000 was obtained using a rabbit reticulocyte cell-free translation system and mRNA preparations from rabbit liver. A study of rat Hp biosynthesis [9] reported a precursor polypeptide of M_r 38000 that was obtained by immunoprecipitation of in vitro translation products by anti rat Hp.

We report here the characterization of the primary translation product of the mRNA for rabbit Hp translated in a rabbit reticulocyte cell-free system and subsequently immunoprecipitated by an antiserum to rabbit Hp. Direct chemical evidence is provided for a signal peptide and for the ordering of the α and β chains of Hp within a single polypeptide chain.

Abbreviations: Hp, haptoglobin; PAGE, polyacrylamide gel electrophoresis; Pth, phenylthiohydantoin; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate

2. MATERIALS AND METHODS

Reagents used in automated sequencing, PAGE components, and HPLC solvents were purchased as in [10]. Radiolabelled amino acids were obtain-

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ed from New England Nuclear and Amersham, and were of the highest specific activity available.

The purification of rabbit Hp, characterization by PAGE, and isolation of the component chains were described in [6]. Automated protein sequence analysis was performed as detailed in [10]. Residues obtained from the 890B Beckman sequenator were identified by HPLC employing C18 reversed-phase chromatography, by gas-liquid chromatography, and by amino acid analysis after back-hydrolysis of the Pth amino acids [10].

Monospecific antiserum to purified rabbit Hp was prepared in a sheep. The specificity of the antiserum was tested by Ouchterlony double diffusion and by immunoelectrophoresis.

The isolation of mRNA from the livers of young control rabbits (~1 kg), and from the livers of similar rabbits injected subcutaneously with turpentine to stimulate the synthesis of Hp [11] was achieved by phenol extraction [12] followed by column chromatography on oligo(dT)cellulose and fractionation on sucrose gradients [13,14]. The mRNA was translated in vitro using the rabbit reticulocyte lysate system [15]. Translation mixtures contained either 900 μCi of [^{35}S]methionine, 450 μCi of [^{35}S]cysteine, or 100 μCi of the other common amino acids labelled with tritium, and 40 μg of the mRNA fractions/ml. Translations were carried out at 30°C for 90 min. The putative translation product of the mRNA for Hp was double-immunoprecipitated [16] with 40 μl of the antiserum to rabbit Hp and 1.2 ml of a rabbit antiserum to sheep IgG/ml of translation mixture. Radiolabelled precursor Hp obtained by double-immunoprecipitation was subjected to automated sequence analysis essentially as in [17]. Pth amino acids eluted by HPLC were pooled, dried and analyzed for radioactivity.

3. RESULTS

Immunoelectrophoresis of purified Hp revealed a single immune precipitate when reacted against either anti rabbit Hp or anti rabbit sera. Analysis by Ouchterlony double diffusion of the antiserum to rabbit haptoglobin tested against the isolated α and β chains of rabbit Hp revealed a reaction of partial identity between the β chain and whole Hp but gave no reaction against the α chain (fig.1). The antigenic difference(s) between the β chain and intact Hp as evidenced by spur formation was most likely due to conformational antibodies.

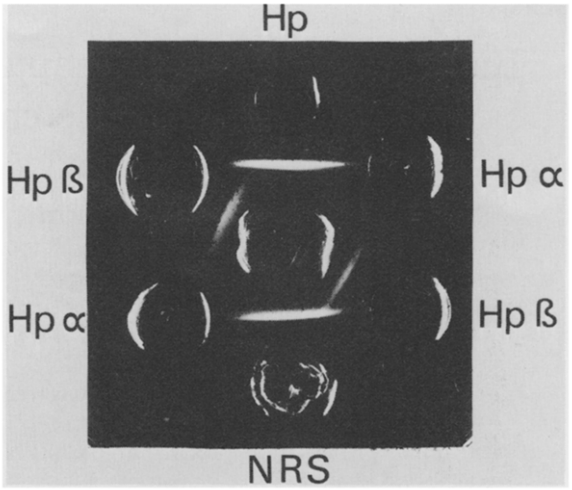

Fig.1. Results of analysis by Ouchterlony double diffusion of purified rabbit Hp, normal rabbit serum (NRS) and the isolated α and β chains of rabbit Hp tested against sheep anti rabbit Hp antiserum.

Analysis by SDS–PAGE and radioautography (fig.2) revealed that the reticulocyte lysate system incorporated [^{35}S]methionine into many polypeptides of a wide range of M_r when programmed by liver mRNA fractions from control (slot 1) or turpentine-treated (slot 2) rabbits. Importantly, gel electrophoresis (slot 3) of the protein immunoprecipitated by rabbit Hp antiserum after translation by the mRNA fraction from the rabbit treated with turpentine demonstrated only one radioactive band of M_r 41 000. No radioactive products were detected following precipitation by the control pre-immune sheep serum (slot 4). About 8-times as much radioactivity was present in the M_r 41 000 putative precursor of Hp when mRNA from turpentine-treated animals (killed 24 h after injection) was used to program protein synthesis when compared to control translations (average of 3 separate expts).

The results of sequence analysis of the NH_2-terminal region of purified α chain of rabbit Hp are given in table 1. Only a single amino acid sequence was obtained. Automated sequence analysis of the putative precursor of rabbit Hp obtained by double-immunoprecipitation demonstrated a single sequence of radiolabelled residues

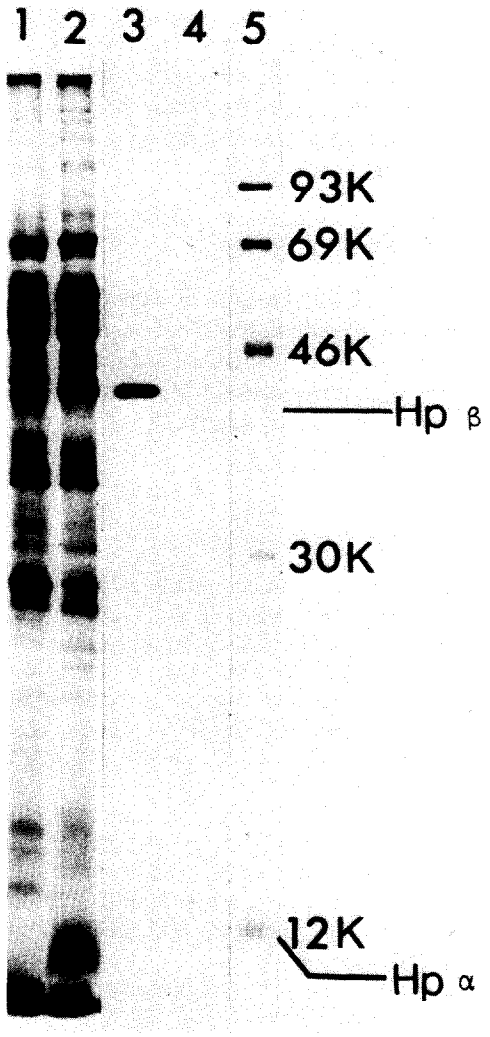

Fig.2. Radioautography of an SDS–PAGE (12.5% gel, reducing conditions) of the [^{35}S]Met containing polypeptides synthesized in the reticulocyte lysate system: slot 1, liver mRNA translation from a control rabbit; slot 2, liver mRNA translation from a rabbit injected with turpentine; slot 3, immunoprecipitate of translation mixture shown in slot 2; slot 4, same as slot 3 but sheep anti-rabbit Hp replaced by pre-immune sheep serum; slot 5, ^{14}C-labelled M_r markers. The positions of α and β chains of rabbit Hp in the gel system used are indicated. The electrophoretic migration of the α chain is anomalously slow [6].

as summarized in table 1. Fig.3 is representative of the occurrence of radiolabelled Pth amino acids as a function of sequencer cycle. Not all of the signal

Table 1

Results of automated sequence analysis of nascent Hp and isolated Hp α chain of rabbit

Edman cycle	Precursor Hp[a]	Edman cycle	Precursor Hp	Edman cycle	Hp α chain
1	Met	19	Ala	1	Ala
2	–[b]	20	–	2	Asp
3	Ala	21	Phe	3	Phe
4	Leu	22	–	4	Gly
5	–	23	Asn	5	Asn
6	Ala	24	–	6	Glu
7	Val	25	Val	7	Val
8	Ile	26	–	8	Thr
9	–	27	–	9	Asp
10	Leu	28	–	10	Ile
11	Leu	29	–	11	Ala
12	Leu	30	–	12	Asp
13	–	31	–	13	Asp
14	–	32	–	14	Ser
15	Gln	33	Cys	15	Cys
16	Leu				
17	Phe				
18	Ala				

[a] Obtained from reticulocyte cell-free system by immunoprecipitation with sheep anti-rabbit Hp

[b] Undetermined

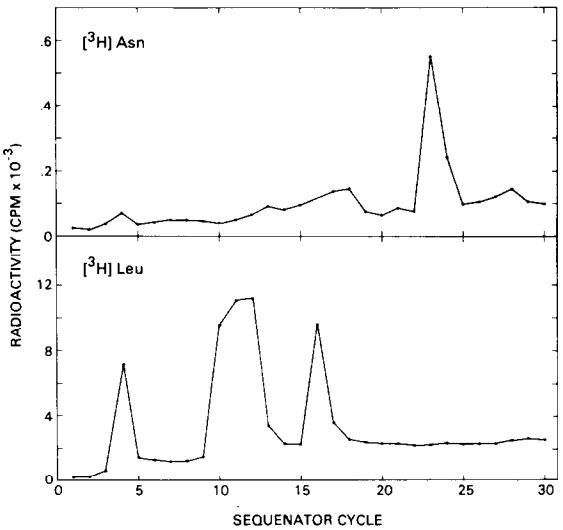

Fig.3. Representative automated sequence analysis of one immunoprecipitation of a reticulocyte lysate translation mixture containing putative precursor Hp labelled with [^{3}H]Leu and [^{3}H]Asn.

region could be assigned at this time due to small amounts of immunoprecipitates and low levels of [^{3}H]amino acid incorporation coupled in some cases with poor sequenator/HPLC recovery of certain amino acids as, for example, threonine, serine or histidine. Low incorporation was also a factor in not obtaining identification of [^{3}H]residues further than position 25 of the putative precursor. This was not the case, however, with [^{35}S]cysteine which incorporated a relatively high level of radioactivity and allowed unambiguous identification of a cysteinyl residue at position 33 of the putative precursor.

4. DISCUSSION

The sequence analyses of the cell-free translation product resulting from immunoprecipitation gave a single sequence that corresponded initially to that of a typical leader sequence [20]. Strikingly, the leader sequence ended at position 18 and was immediately followed by the α chain sequence as established by sequence analysis of isolated Hp α chain. Residues Ala-19, Phe-21, Asn-23, Val-25 and the cysteinyl residue at position 33 of the translation product were in identical sequence to residues 1, 3, 5, 7 and 15 of purified α chain of rabbit Hp (table 1). Since the translation product was precipitated by an antiserum that reacted only with intact Hp and with the β chain, but not with the α chain (fig.1), these results gave strong evidence that rabbit Hp was synthesized as a single chain containing both α and β chain components. Further evidence to support this conclusion was the fact that only a single translation product was obtained using the anti Hp antiserum (fig.2). Moreover, the M_r of 41000 determined for the precursor of Hp is reasonably close to the calculated M_r of about 38000 that includes the leader sequence (~2000), the α chain (9200 [1]), and the β chain (27000 without carbohydrate [1]). The small increase in M_r of the precursor when compared to the summation M_r of the component chains may be due to the presence of some residues (< 30) between the α and β regions that are excised by limited proteolysis.

These results further emphasize the chemical similarity previously noted [1] between Hp and the chymotrypsinogen family of serine proteases. Like Hp the serine proteases are typically synthesized as single chain precursors and are subsequently processed by limited proteolysis. In some cases this proteolysis leads to multichained serine proteases. In this regard, Hp is similar to Factor X1 which is cleaved by limited proteolysis to give a tetrachain serine protease.

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