Determination of the Subunit Composition of Haptoglobin 2-1 Polymers Using Quantitative Densitometry of Polyacrylamide Gels

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Human haptoglobin (Hp), a hemoglobin-binding glycoprotein containing two types of polypeptide chains, α and β, in equimolar amounts linked by disulfide bonds, exists in three major phenotypes determined by the properties of the α chain: Hp 1-1 (α'), Hp 2-2 (α'), and Hp 2-1 (α' and α'). Hp 2-2 and Hp 2-1 form a series of α-disulfide-linked polymers. The subunit composition of the Hp 2-1 series was studied by isolation of single Hp 2-1 polymers by polyacrylamide gel electrophoresis. After reductive disulfide cleavage and alkylation the relative content of α' and α polypeptide chains was determined by quantitative densitometry of acid/urea polyacrylamide gels stained with Coomassie brilliant blue R250. The molar ratios α'/α for the Hp 2-1 polymers, P1 through P5 (in order of decreasing electrophoretic mobility), were found to be: P1, 0.0 (α' only); P2, 0.48; P3, 0.97; P4, 1.6; P5, 2.0.

Since one α'-Hb half-molecule is known to bind to each Hp β chain, the β polypeptide chain content of each of the Hp 2-1 polymers could be estimated by counting the number of Coomassie blue bands formed after electrophoresis of isolated Hp 2-1 polymers fractionally saturated with cyanmethemoglobin (Hb). The number of β chains present in Hp 2-1 polymers P1 through P4 was determined to be: P1, 2; P2, 3; P3, 4; and P4, 5.

Molecular weights of the Hp 2-1 polymers were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using as standards the almost homologous Hp 2-2 polymer series whose molecular weights are known from ultracentrifugation studies. Molecular weights for the first five Hp 2-1 polymers were estimated to be 107,000; 162,000; 217,000; 274,000; and 331,000, respectively.

These data are consistent with the previously proposed model for the subunit composition of the Hp 2-1 polymer series where P1 = (α'β)2, and the subsequent polymers in order are represented as (α'β)2(αβ)n, where n = 1, 2, 3, 4, . . .

Haptoglobin (Hp) is a hemoglobin-binding α2 glycprotein present in the serum of most mammalian species. In the human it is polymorphic and is commonly found in one of three genetically determined phenotypes, Hp1-1, Hp2-1, and Hp2-2. These types were easily identified by electrophoresis at alkaline pH initially in starch gels (1) and later in polyacrylamide gels (2). Gel electrophoresis of these proteins at acid pH in the presence of denaturing agents (8 M urea, 6 M guanidine) after disulfide reduction and alkylation yielded two types of polypeptide chains designated α and β (3). The β chains contain all of the carbohydrate moiety (4) and in all three phenotypes are identical by electrophoresis under varying conditions (3), amino acid composition (4), peptide mapping (5), and in having a molecular weight of 40,000 (4). The α chains, however, are of two main forms: α of molecular weight 9,067 and α of molecular weight 16,685 (4, 6). Amino acid sequence studies have shown that the α chain is a partial reduplication of the α chain (7, 8). Hp 1-1 contains only α' chains, Hp 2-2 has only α' chains, but Hp 2-1 has both types of α chains (3).

Hp 2-1 and Hp 2-2 each form a stable, distinct series of polymers which are detected on gel electrophoresis as a series of bands of decreasing mobility and concentration, whereas Hp 1-1 migrates as a single band (1). The Hp 1-1 molecule has been shown to have a molecular weight of 100,000 and to consist of two α' and two β chains linked by disulfide bonds (3, 4, 9). The polymers of Hp 2-1 and Hp 2-2 are also linked by disulfide bonds (3, 10-12), but their relative and absolute composition of α and β chains has been subject to speculation (3-22). Recently the subunit composition of the first six polymers of the Hp 2-2 series has been elucidated. These isolated polymers were shown to have equimolar amounts of α' and β chains, and molecular weights consistent with a polymer series (α'β)n, where n = 3, 4, 5, . . . (12).

The more complex subunit composition of the Hp 2-1 polymer series, however, has not been directly elucidated. Most notably lacking has been information on the relative
content of $\alpha^1$ and $\alpha^2$ polypeptide chains in each Hp 2-1 polymer, the information most helpful in differentiating among the various previously proposed models of the subunit composition of the Hp 2-1 series (13-22). In an attempt to clarify these points, we have isolated the first five polymers of the Hp 2-1 series in electrophoretically pure form by elution from polycrylamide gels and after reductive cleavage and alkylation have analyzed their chain composition by quantitative densitometry of acid/urea polycrylamide gels stained with Coomassie brilliant blue R250. The $\beta$ chain composition was determined by analysis of the numbers of intermediates formed when each Hp 2-1 polymer was mixed with sub saturating amounts of cyanmethemoglobin (Hb), based on the previous findings that it is the $\beta$ chain which is responsible for the hemoglobin-binding properties of the Hp molecule (23). Molecular weight estimations for the polymers of the Hp 2-1 series were performed by polycrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate.

**EXPERIMENTAL PROCEDURE**

**Purification of Hp 2-1 and Hp 2-2—Hp 2-1 was obtained from the National Institutes of Health Blood Bank. After centrifugation to remove any residual erythrocytes, purification proceeded by DEAE-cellulose chromatography followed by gel filtration on Sephadex G-200 as previously described (29). Sephadex fractions containing Hp 2-1 polymers 1 through 7 were pooled, and this material was shown to be free of other proteins by analytical polycrylamide gel electrophoresis at 25 $\mu$g of protein per gel. Hp was assayed as hemoglobin-binding capacity (HBC) by the hemoglobin peroxidase activation method of Tarukoski (24), except that the Hp/Hb mixture was decreased to 0.2 ml and the volume transferred to the o-dianisidine reagent was increased to 0.1 ml for increased sensitivity.

Analytical Polycrylamide Gel Electrophoresis—Hp 2-1 was analyzed by the discontinuous polycrylamide gel electrophoresis method of Davis (25), using 7%, 38/l (acrylamide/methylenebisacrylamide) cross-linked separating gels. Gels were stained with 0.05% Coomassie brilliant blue R250 in 12% trichloroacetic acid and destained in 12% CCl$_3$COOH in a manner similar to that previously described (26).

Preparative Polycrylamide Gel Electrophoresis—Hp 2-1 polymers were separated by the vertical slab gel electrophoresis method of Raymond (27) using 6-mm thickness, 7%, 38/l cross-linked gels in a manner similar to that previously described (26).

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Preparative Polycrylamide Gel Electrophoresis—Hp 2-1 polymers were separated by the vertical slab gel electrophoresis method of Raymond (27) using 6-mm thickness, 7%, 38/l cross-linked gels in a continuous buffer system of 0.09 M Tris, 0.09 m boric acid, 0.0024 M EDTA, pH 8.3 (28). Electrophoresis proceeded at 250 V, 0° for 12 h (for isolation of polymers 1 through 3) and 26 h (for isolation of polymers 4 through 5). After electrophoresis two guide strips were cut from the sides of the gel and stained with 0.0125% Coomassie blue G250 in 12% CCl$_3$COOH for 1 h followed by 30 min of destaining in 5% acetic acid (29). After repositioning the guide strips, individual Hp 2-1 polymers were sliced from the gel. These gel pieces, usually about 2 to 3 cm$^2$, were then macerated and suspended in 30 to 50 ml of the Tris/boric acid/EDTA buffer, pH 8.3, used in electrophoresis and allowed to elute at 20°C for 2 to 5 days. The eluates were then filtered free of suspended gel particles, dialyzed against deionized water, lyophilized, and resuspended in 0.2 to 1.0 ml of deionized water for analytical polycrylamide gel electrophoresis and assay of hemoglobin-binding capacity. The yield ranged from 25 to 75%. Only those eluates containing single, electrophoretically pure Hp 2-1 polymers were used.

Separation of $\alpha^1$, $\alpha^2$, and $\beta$ Polypeptide Chains—Approximately 500 $\mu$g (0.016 $\mu$mol of $\alpha$ subunits) of each isolated Hp 2-1 polymer was incubated at 20°C for 30 min in a standard reaction mixture containing 8 $\mu$M urea and sodium formate buffer, pH 3.2, in a discontinuous system previously described (30). Electrophoresis proceeded for 3 h at a constant current of 2.5 mA per gel.

Quantitative Densitometry—Acid/urea gels containing separated $\alpha^1$, $\alpha^2$, and $\beta$ chains from the Hp 2-1 polymers were stained with 0.05% Coomassie brilliant blue R250 in 12% CCl$_3$COOH for 12 h, followed by destaining in 12% CCl$_3$COOH for 24 h. The gels were then scanned at 570 nm in a Gilford model 240 spectrophotometer. Optical density was recorded on a Varian model G-2010 chart recorder coupled to a Disc model 610 automatic integrator, which was manually triggered to obtain the areas beneath the optical density tracings of the $\alpha^1$ and $\alpha^2$ bands. The integrated area values for the separated $\alpha^1$ and $\alpha^2$ band tracings were converted to mole equivalents by division by their respective molecular weights (9,057 for $\alpha^1$, and 16,685 for $\alpha^2$, based on amino acid composition and ultracentrifugation studies (4, 6)) and the mole ratio $\alpha^2/\alpha^1$ was computed. Duplicate or triplicate gels were run for all quantitative studies.

**Fractional Hb Saturation of Hp 2-1 Polymers—Cyanmethemoglobin was prepared and mixed in subsaturating amounts with isolated Hp 2-1 polymers as previously described (22). The fractionally saturated Hp-Hb complexes were separated electrophoretically by the standard analytical polycrylamide gel procedure.

**Molecular Weight Estimations**—Molecular weight estimations were performed by continuous vertical slab gel electrophoresis using gels with various concentrations of acrylamide at a constant 38/l cross-linking in the presence of 0.1% sodium dodecyl sulfate and 0.05 M sodium phosphate buffer, pH 7.1, in a procedure similar to that described by Shapito et al. (31) and Weber and Osborn (32). Samples were incubated for 2 h at 37°C in 1% sodium dodecyl sulfate prior to electrophoresis. Electrophoresis proceeded at 20°, 100 V, for 4 to 8 h depending on gel concentration. Molecular weight standards included bovine serum albumin (Sigma), human $\gamma$-globulin (Mann), human fibrinogen (gift of Dr. John Finlayson), and the purified Hp 2-2 polymer series whose molecular weights have recently been determined (12).

**RESULTS**

Accurate determination of the relative content of $\alpha^1$ and $\alpha^2$ polypeptide chains in the Hp 2-1 polymers requires quantitative release of the $\alpha^1$ and $\alpha^2$ chains by reduction of the disulfide bonds of the Hp molecule and alkylation to prevent disulfide rearrangement or interchain exchange as well as an accurate densitometric measurement of the amount of each chain. In the standard reductive cleavage and alkylation procedure diethiothreitol was present in 400-fold molar excess relative to Hp 2-1, and Fig. 1 illustrates that even 90% of this amount of diethiothreitol resulted in no significant change in the amount of $\alpha$ chain determined by gel densitometry or in the $\alpha^2/\alpha^1$ mole ratio. The $\alpha$ chain yield was also unaffected when the standard amount of iodoacetamide was decreased from 2.5 molar excess to approximate molar equivalence relative to diethiothreitol (data not presented). Increasing the incubation time in diethiothreitol from the standard 30 min to 6 h or increasing the subsequent...
incubation period after the addition of iodoacetamide from the standard 30 min to 2 h resulted in slight decreases in a chain yield, but no significant change in the mole ratio a'/a' (Fig. 2). Thus, our conditions of reductive disulfide cleavage and alkylation appeared adequate to provide quantitative release of a^2 and a^1 polypeptide chains.

In order to evaluate the accuracy of densitometric measurements on the gels, reduced and alkylated Hp 2-1, unseparated into individual polymers, was applied to acid/urea polyacrylamide gels and the areas beneath the a^2 and a^1 bands were measured. The sum of the areas beneath the densitometric tracings of the a^2 and a^1 bands was a linear function of the amount of protein added per gel within the range of 6 to 20 μg of protein (measured as HBC) (Fig. 3A). The a^2/a^1 mole ratio was constant over this range (Fig. 3B) and only gels falling within these limits were used to compute the a^2/a^1 mole ratios for the isolated Hp 2-1 polymers. Plots of the areas for a^2 and a^1 bands considered separately were also linear when graphed against total protein added per gel, but their slopes were not equal because the microgram amounts of a^2 and a^1 chains represented different fractions of the molecular weight of the Hp molecule (Fig. 3B). These fractions, however, may be estimated and were found to be 0.144 for a^2 and 0.0942 for a^1, based on the known molar equivalence of the total a and total β chain content (33), the known molecular weights of the a^2, a^1, and β chains (4), and the observed mole ratio a^2/a^1 presented below. With the abscissa thus recalculated the plots of the areas of the a^2 and a^1 bands versus micrograms of a chain added per gel were linear over the same range and had the same slope (Fig. 3C). When these same curves were plotted using values of the mole ratio varying by greater than 10% from the observed value of 0.83 (see below), the slopes of the curves for a^2 and a^1 were no longer equal, suggesting that the densitometric estimation of the relative a^2 and a^1 chain content was in error by no more than 10%. These findings indicate that the a^1 and a^2 chains have equivalent staining properties, as would be expected for two polypeptide chains of almost identical percentage amino acid compositions (4).

Table I presents the results of several separate determinations of the mole ratio of a^2 to a^1 chains for unseparated Hp 2-1 and for isolated Hp 2-1 polymers 1 through 5. All ratios were determined in duplicate or triplicate over a range of protein concentrations, and the a^2 and a^1 bands were clearly separated in all cases (Fig. 4). Polymer 1 yielded only a^1 chains, as has been previously documented (22). The mole ratio of a^2/a^1 corresponds closely to that predicted from the previously proposed model for the Hp 2-1 polymer series (15, 21, 22) where the first polymer, like Hp 1-1, contains two a^1β subunits followed by increments of one additional a^1β subunit for each succeeding polymer, as diagrammed in Fig. 5.

The relative β chain composition of the Hp 2-1 polymers could not be determined by gel densitometry because of its quantitatively different staining properties with Coomassie blue and because after reductive cleavage and alkylation under various conditions it migrates as a series of bands in acid/urea gels as has been noted by Smithies et al. (3).

Hemoglobin binding by haptoglobin is a function of the β polypeptide chain (23), and it has been shown that one a^2 half molecule of hemoglobin will bind for each β chain of Hp...
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1-1 (34-38). Hp 2-1 and Hp 2-2 have also been shown to bind amounts of hemoglobin equivalent to Hp 1-1 based on heme and nitrogen contents (39, 40). When hemoglobin is mixed in subsaturating amounts with Hp 1-1, three Coomassie blue bands are distinguishable after electrophoresis; these bands correspond to free Hp, Hp fully saturated with hemoglobin, and Hp fractionally saturated with hemoglobin (22, 38). Thus, the number of Coomassie blue bands (n) is one more than the number of β chains (n - 1) present. The number of Coomassie blue bands formed after mixing of the isolated Hp 2-1 polymers 1 through 4 with subsaturating amounts of hemoglobin is listed in Table II. These results confirm those previously reported (22) and are consistent with the proposed model for the subunit composition of the Hp 2-1 polymer series.

The results of polyacrylamide gel electrophoresis of the Hp 2-1 polymer series in the presence of 0.1% sodium dodecyl sulfate is illustrated in Fig. 3. The Hp 2-2 polymer series of molecular weight standards fall on a smooth curve (which at low gel concentrations is a straight line) with electrophoretic mobilities lower than those for the other protein standards. This result may be due to the high (19.4%) carbohydrate content of the Hp β chain (4) and to the presence of several interchain and intrachain disulfide bonds in the Hp polymers (41, 42), properties which have been shown to reduce sodium dodecyl sulfate binding to proteins (43, 44). Because the Hp 2-2 and Hp 2-1 polymer series have identical carbohydrate composition (4, 45) and almost identical polypeptide chain composition, the proteins of the Hp 2-2 series, whose molecular weights are known from the sedimentation equilibrium studies of Fuller et al. (12), may be used as standards to estimate the molecular weights of the Hp 2-1 series. The tentative molecular weight estimations of the Hp 2-1 polymer series determined in this manner over a range of gel concentrations are listed in Table III, and can be seen to be consistent with the proposed model.

DISCUSSION

Satisfactory quantitation of proteins by densitometry of polyacrylamide gels stained with Coomassie blue R250, the stain used in this study, has been demonstrated by Fishbein for the polymers of jackbean urease, provided the range of protein applied to each gel and the distance of migration were suitably restricted (46). Because the Hp α2 chain is a partial reduplication of the α1 chain with an essentially identical percentage amino acid composition (4), it would be anticipated that these two polypeptide chains should stain equivalently with Coomassie blue, an anionic dye which binds to NH2+ groups on proteins (47). No attempt was made to determine directly the specific stain uptake for the α2 and α1 chains because no independent assessment of the absolute amounts of α2 and α1 was available. We have shown that the densitometrically determined amount of stain bound is linear for the separate α2 and α1 bands from each reductively cleaved

![Fig. 3](http://www.jbc.org/)

**Fig. 3.** Quantitative densitometry of reduced, alkylated whole Hp 2-1 α2 and α1 chains on acid/urea-polyacrylamide gels stained with Coomassie blue. Linear relationship between total protein added per gel (as HBC) and A, sum of areas beneath densitometric tracings of α2 and α1 chains (O) and B, areas beneath the densitometric tracings of individual α2 (●) and α1 (□) chains. Note that the mole ratio α2/α1 (▲) remains essentially constant over the range of 6 to 20 µg of protein/gel. C, equivalent densitometric response of α2 and α1 bands when micrograms of α chain are recalculated.2

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Measured molecular weight ± S.D.</th>
<th>Theoretical molecular weight</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>107,000 ± 4,200 (6)</td>
<td>100,000</td>
</tr>
<tr>
<td>2</td>
<td>162,000 ± 4,400 (11)</td>
<td>155,000</td>
</tr>
<tr>
<td>3</td>
<td>217,000 ± 3,200 (11)</td>
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<tr>
<td>4</td>
<td>974,000 ± 6,800 (11)</td>
<td>968,000</td>
</tr>
<tr>
<td>5</td>
<td>331,000 ± 5,100 (8)</td>
<td>324,000</td>
</tr>
</tbody>
</table>

* Number of determinations.
and alkylated Hp 2-1 polymer could be quantitatively scanned on the same gel, reproducible, direct determinations of the relative amounts of these two polypeptide chains could be obtained. The conditions of reductive disulfide cleavage and alkylation also appeared to be quantitative.

The densitometrically determined relative composition of haptoglobin and hemoglobin in the series of bands formed when the Hp 2-1 polymers 1 through 3 are mixed with subsaturating amounts of hemoglobin has been shown by Pastewka et al. (22) to be consistent with the stepwise binding of hemoglobin α8 half-molecules for each Hp α3 subunit, as has been well documented for Hp 1-1 (34-38). The Hp β chain...
has been shown to be the hemoglobin-binding unit (23). The number of αβ subunits for each Hp 2-1 polymer should then correspond to one less than the number of Coomassie blue bands (subtracting the free Hp band) formed by electrophoresis of Hp fractionally saturated with hemoglobin. Our data on the number of haptoglobin-hemoglobin complexes for Hp 2-1 polymers 1 through 4 confirm those previously reported by Pastewka et al. (22). This method has also been applied by these authors to the Hp 2-2 polymer series, and their data on the number of αβ subunits in the first three polymers in this series is consistent with the recent molecular weight determinations performed by Fuller et al. (12).

The Hp polymers migrate more slowly on sodium dodecyl sulfate-polyacrylamide gels relative to other standard molecular weight markers, presumably at least in part due to their carbohydrate composition and the presence of multiple disulfide linkages (43, 44). Similar anomalous electrophoretic behavior in sodium dodecyl sulfate has been observed with Hp 1-1 (42). We were unable to confirm the finding by Fuller et al. (12) that the Hp 2-2 polymers fell on the same curve as bovine serum albumin, human γ-globulin, bovine fibrinogen, and lobster fibrinogen. At appropriate acrylamide concentrations, the Hp 2-2 polymers fall on a straight line and extrapolation is necessary only to estimate the molecular weight of the first Hp 2-1 polymer, which migrates identically to Hp 1-1, whose molecular weight has been shown to be 100,000 by several authors (9, 34, 35, 48), a value consistent with our estimate. Use of the Hp 2-2 series to estimate the molecular weights of the Hp 2-1 polymer series provides a uniquely homologous set of standards for molecular weight determination of these multichain glycoproteins with almost identical carbohydrate and disulfide composition. The accuracy of these estimations depends on the reliability of the molecular weights of the Hp 2-2 series as determined by sedimentation equilibrium by Fuller et al. (12), whose values had a precision of 2.5 to 8.3%.

That sodium dodecyl sulfate-polyacrylamide gel electrophoresis may be a valid method for estimation of the molecular weights of the Hp 2-1 polymer series in this circumstance is further suggested by the findings of Ogawa et al. (20) on the molecular weights of the saturated Hp-Hb complexes of the three Hp 2-1 polymers. Using ultracentrifugation and diffusion studies these authors found the molecular weights for the saturated Hp-Hb complexes to be 169,000 for P1; 245,000 for P2; and 342,000 for P3. Subtracting the portion contributed by the Hp half-molecule binding to each β chain yields molecular weights of 104,500; 148,250; and 213,000, respectively, for the first three Hp 2-1 polymers. These values correspond closely to our data and to the values predicted by the model for Hp 2-1 polymer series.

Our data on the mole ratios αβ/α′ for the first five Hp 2-1 polymers, taken together with the indirect determination of the number of β chains by enumeration of the fractionally saturated Hp-Hb intermediates and with the molecular weight estimations for the Hp 2-1 polymers, strongly support the model for the subunit composition of the Hp 2-1 polymer series as first suggested in part by Sutton and Karp (15) and Sutton (21) and elaborated by Pastewka et al. (22), where polymer 1 = (α′β)α polymer 2 = (αβ)α(αβ)α, polymer 3 = (αβ)α(αβ)α, polymer 4 = (αβ)α(αβ)α, and polymer 5 = (αβ)α(αβ)α. The earliest model for the Hp 2-1 polymers proposed by Allison (13) antedated the discovery of the Hp α and β chains; this information was also not incorporated into the later models proposed by Marinis and Ott (16) and by Ogawa and co-workers (19, 20). The model proposed by Shim et al. (17) where the Hp 2-1 series is represented by (αβ)α(αβ)α with n = 0, 2, 4, 6 . . . is inconsistent with our data.

Although our data do not allow assignment of the relative positions of the αβ and α′α′ subunits in each Hp 2-1 polymer, we nevertheless feel that the most likely structure is that represented in Fig. 5, where the αβ subunits constitute the ends of each polymer in the series (αβ)α(αβ)α n = 0, 1, 2, 3 . . . . This arrangement is suggested by the known compositions of Hp 1-1 ((αβ)α) and the Hp 2-2 polymer series ((αβ)α (n = 3, 4, 5 . . . ) and the recent assignment of interchain disulfide linkages in the three Hp phenotypes by Malchy and co-workers (41, 49). Working with cyanogen bromide cleavage peptides which leave the methionine-free α chain intact, these authors found a 21α-21α disulfide in fragments from Hp 1-1. Similar fragments from Hp 2-1 and Hp 2-2 existed as a series of polymers, indicating that the half-cystine at position 21 in the α chain, which with partial reduplication results in half-cystines at positions 21 and 80 in the α chain, is responsible for the Hp 2-1 and Hp 2-2 polymorphic series. The other α chain half-cystines appeared to be involved in intra-α chain linkages or α chain linkages with no evidence for ββ disulfides. Thus, each αβ subunit would have 1 half-cystine involved in an interchain disulfide linkage, whereas each α′α′ subunit would have 2 such half-cystines to link with other subunits, allowing for polymerization to proceed until terminated by the addition of an αβ subunit.

For Hp 2-1 unseparated into individual polymers Black et al. (4) found αα′ and α′α′ chains to be present in approximately equimolar amounts. Our measured mole ratio αβ/α′α′ of 0.85 for Hp 2-1 is not inconsistent with their findings, but the minor discrepancy could be accounted for if our initial purification procedure yielded relatively less of the higher molecular weight Hp 2-1 polymers. That our estimations of the molar α′/α′ ratios for the separated Hp 2-1 polymers fit closely in stepwise fashion over a greater than 4-fold range with the model for each successive polymer suggests that our method of quantitative densitometry of polyacrylamide gels is a reliable one and is unlikely to account for the small deviation of our data from those of Black et al. (4).

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