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Subunit Composition of Haptoglobin 2-2 Polymers†

Gerald M. Fuller,* Marilyn A. Rasco, Michael L. McCombs,‡ Donald R. Barnett, and Barbara H. Bowman

ABSTRACT: Hp 2-2 forms a series of discrete polymers of increasing molecular weight. In the present study Hp 2-2 polymers have been isolated from each other by polyacrylamide gel electrophoresis. Quantitative amino-terminal analysis and amino acid analysis provide evidence that each polymer consists of $\alpha 2$ and β polypeptide chains in a 1:1 ratio. Molecular weight estimations by sodium dodecyl sulfate acrylamide gel electrophoresis and by ultracentrifugational analysis indicates that each polymer differs from the next member of the series by an average increment 54,500. This approximates a subunit consisting of an $\alpha 2$ and a β polypeptide chain. Experimental results indicate that the $\alpha 2/\beta$ subunits are linked together through disulfide bonds to form each polymer.

Haptoglobin (Hp)[1] is an α_2 globulin in human serum whose function is to bind free hemoglobin (Hb). Three common genetic types have been described on the basis of their electrophoretic migration patterns on vertical starch gel at alkaline pH (Smithies, 1955). More recently, polyacrylamide gel electrophoresis has demonstrated similar patterns (Raymond, 1962; Woodworth and Clark, 1967; McCombs and Bowman, 1970). An interesting feature of haptoglobin types 2-2 and 2-1 is their capacity to form a series of discrete stable polymers. During electrophoresis Hp 1-1 migrates as a single band, whereas Hp 2-2 and 2-1 each forms a series of polymeric bands of decreasing mobility and concentration.

The Hp molecule is comprised of two pairs of nonidentical polypeptide chains, designated α and β, which are held together by disulfide bonds (Smithies *et al.*, 1962). The α chain, which exists in two common phenotypes, is responsible for the genetic polymorphism observed in Hp. Hp 1-1 contains $\alpha 1$ chains, each of which has a molecular weight of 9000, while Hp 2-2 contains $\alpha 2$ chains, each of which has a molecular weight of 17,300 (Dixon, 1966). Both $\alpha 1$ and $\alpha 2$ chains are present in Hp 2-1. The β chain, 40,000 molecular weight, is identical in all three types of haptoglobins (Shim *et al.*, 1965).

There have been several different views concerning the number of α and β polypeptide chains which occur in the polymers of Hp 2-1 and Hp 2-2. By observing the number of bands on acrylamide gels of partially saturated Hp–Hb complexes, Sutton (1970) considered that each successively larger polymer was due to the addition of one β chain. The presence of α chains in proportion to β chains was suggested, but no experimental verification for this was given. An earlier hypothesis of Allison (1959) was that each successively larger polymer resulted from addition of one monomeric molecule. The number of chains of this monomer was unspecified. Parker and Bearn (1963) and Shim and Bearn (1964) proposed an addition of two α chains and two β chains as the increment between consecutive polymers. Marnay (1961) proposed that the difference between polymer size was due to the addition of half-molecules. Although the number of polypeptide chains was unspecified, this presumably had reference to α/β increments. Marinis and Ott (1965) proposed that each polymer is a dimer of the preceding smaller polymer.

In the present study the Hp 2-2 polymers (one through six) have been isolated by polyacrylamide gel electrophoresis

† From the Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas 77550. *Received May 18, 1972.* This work was supported in part by grants from the National Institutes of Health (HD 03321) and the Robert A. Welch Foundation (H-378).

‡ Present address: Department of Medicine, Section of Hematology and Immunology, University of California, San Francisco, Calif. 94122.

[1] Abbreviations used are: Hp, haptoglobin; Hb, hemoglobin; β-ME, β-mercaptoethanol; Pth, phenylthiohydantoin derivative.

(Fuller *et al.*, 1971). An investigation of the isolated polymers provides direct experimental evidence that adjacent Hp polymers on acrylamide gels differ by one $\alpha 2/\beta$ increment. With increasing concentrations of β-ME, the polymers dissociated into smaller molecular species indicating the importance of disulfide linkage in polymer formation. The intrapolymer disulfide linkage is especially interesting in view of the fact that no free titratable SH groups have been detected in the haptoglobin molecule (Tattrie and Connell, 1967).

Materials and Methods

Purification of Haptoglobin. Haptoglobin 2-2 was purified from ascites fluid taken from a patient with an ovarian tumor. This fluid was chosen rather than serum because it contained unusually high concentrations of haptoglobin (Laurell, 1959). Results from structural studies have demonstrated that haptoglobin from an individual's serum and ascites fluid are identical (Barnett *et al.*, 1972). Haptoglobin was purified from this material following the procedure described by Gordon *et al.* (1968).

Analytical Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed at 400 V (constant voltage) in an 18 × 13 × 0.3 cm vertical slab gel electrophoresis apparatus. The gels were 5% Cyanogum-41 (Fisher Scientific) catalyzed by *N,N,N',N'*-tetramethylethylenediamine. A continuous pH buffer system consisted of 0.1 M Tris–0.09 M boric acid (pH 8.4). The gels were stained with 0.01% Commassie Brilliant Blue in 15% acetic acid for 1–2 hr and destained with 7% acetic acid.

Preparative Acrylamide Gel Electrophoresis. Haptoglobin polymers were isolated by preparative polyacrylamide electrophoresis in an apparatus similar to the one described above except the gel thickness was increased to 0.6 cm. The buffer system was identical to that described for the analytical gels. The electrophoresis time was 4400 V-hr (*ca.* 220 V for 20 hr). The gel was removed after electrophoresis and two 1-cm guide strips were cut the length of the gel and stained. The stained strips were then repositioned with the acrylamide slab so that individual polymers could be cut from the gel. It was usually possible to obtain six to eight polymers which did not overlap each other. Each acrylamide strip containing a separated polymer was sealed in dialysis tubing and suspended vertically in an electrophoresis chamber. Cross-electrophoresis (25 V, 400 mA) was carried out in a 0.05 M Tris–borate buffer (pH 8.4) at 15° for approximately 24 hr. Following electrophoresis the acrylamide strips were removed from the dialysis bag which contained the isolated polymer in solution.

Sodium Dodecyl Sulfate Polyacrylamide Gels. Polyacrylamide gel electrophoresis containing the detergent sodium dodecyl sulfate was utilized to estimate the molecular weight of each isolated polymer. The analytical slab gel apparatus previously described was used. Acrylamide gels (5%) were prepared with 0.05 M sodium phosphate buffer (pH 7.2), containing 0.1% sodium dodecyl sulfate (Weber and Osborn, 1969; Chrombach and Rodbard, 1971). The gels were subjected to electrophoresis for 8 hr at 100 V, removed, and stained. Protein band resolution improved when the gel was washed with two to three changes of 7% acetic acid over an 8-hr period prior to the addition of 0.01% Coomassie stain. The reference proteins used to calculate molecular weights were: lobster fibrinogen, 420,000; bovine fibrinogen, 330,000; human γ-globulins, 160,000; bovine serum albumin, 68,000.

Amino Acid Analysis. Triplicate amino acid analyses were performed on each of four haptoglobin polymers after hydrolysis in 6 N HCl for 22 hr at 110° in sealed, evacuated hydrolysis tubes. A Beckman Amino Acid Analyzer Model 116, equipped with high-sensitivity cuvet and an expanded scale was used. An extinction coefficient, $\epsilon_{280}^{1\%}$, of 12.1 (Hermann-Boussier *et al.*, 1960) was used to estimate protein concentration.

Amino-Terminal Analysis. Quantitative amino-terminal analysis of intact haptoglobin 2-2 and three of the purified polymers was performed using a Beckman Automatic protein sequencer. The Pth derivatives were identified quantitatively by gas chromatography according to the single-column method of Pisano and Bronzert (1969, 1970), using a Nuclear-Chicago, Series 5000, gas chromatograph. Quantitation was based on detector response to known quantities of authentic Pth-valine and Pth-isoleucine (Pierce Chemicals).

Sedimentation Equilibrium. Molecular weights were determined by the high speed equilibrium method of Yphantis (1964), using schlieren optics in a Beckman Model E analytical ultracentrifuge. A six-channel centerpiece was used to determine molecular weights at three different protein concentrations simultaneously. The polymers were dialyzed against the solvent, 0.1 M Tris (pH 7.5), for at least 24 hr prior to the experiment. Sedimentation was performed at 20° for 20–24 hr at speeds sufficient to achieve miniscus depletion. Photographs were taken and the plates read on a Nikon microcomparator.

Molecular weights were calculated from the equation $\ln H - \ln r = (1 - \bar{v}\rho)/2RTMr^2$. The function $\ln H - \ln r$ *vs.* r^2 is linear, where H is the height of the schlieren pattern at a point r centimeters from the axis of rotation. The quantities $\ln H - \ln r$ and r^2 are calculated for each point along the schlieren curve, and by regression analysis the slope obtained was used to determine the molecular weight. The partial specific volume of Hp 2-2 was calculated from the amino acid and carbohydrate composition and a value of 0.718 was obtained.

Results

Isolation of Individual Haptoglobin Polymers. In this study the haptoglobin polymers were identified according to the distance they migrated into a 5% polyacrylamide gel. The most rapidly migrating polymer was called polymer 1, the next polymer 2, etc. The polymer number does not reflect the subunit composition. Usually, 12–14 separated polymers of haptoglobin 2-2 could be seen on a polyacrylamide gel under the conditions described.

Analytical gel electrophoresis of Hp 2-2 polymers separated by preparative gel electrophoresis is shown in Figure 1. Each darkly stained polymer was accompanied by a slower migrating, weakly stained component which was present in every preparation of isolated polymer. Because of the characteristic migration of this minor band and that of the major polymer band, it appears that the minor component is a dimer of the major polymer. The concentration of this minor component did not increase with the age of the preparation, nor after freezing, thawing, or lyophilization.

Molecular Weight Estimations. The molecular weights of six haptoglobin 2-2 polymers were determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and in the analytical ultracentrifuge. Figure 2 illustrates a typical separation of the isolated haptoglobin polymers. Haptoglobin 1-1, a single molecular species with a molecular weight of 98,770 ± 2270 (Cheftal and Moretti, 1966; Moretti *et al.*, 1966), migrates as a single band on sodium dodecyl sulfate acrylamide gel electrophoresis. When its molecular

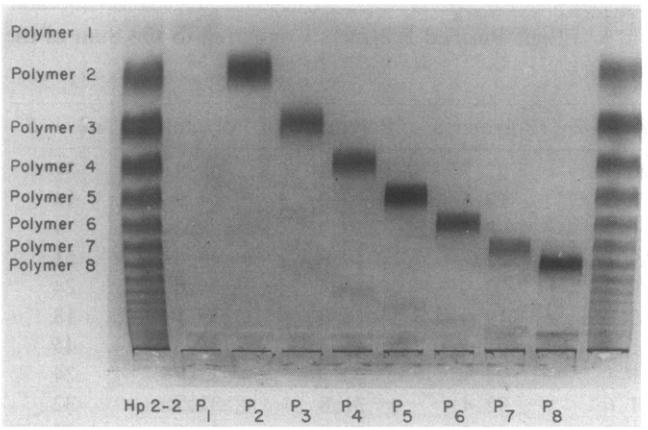

FIGURE 1: Polyacrylamide gel electropherogram of purified haptoglobin 2–2 and isolated haptoglobin 2–2 polymers.

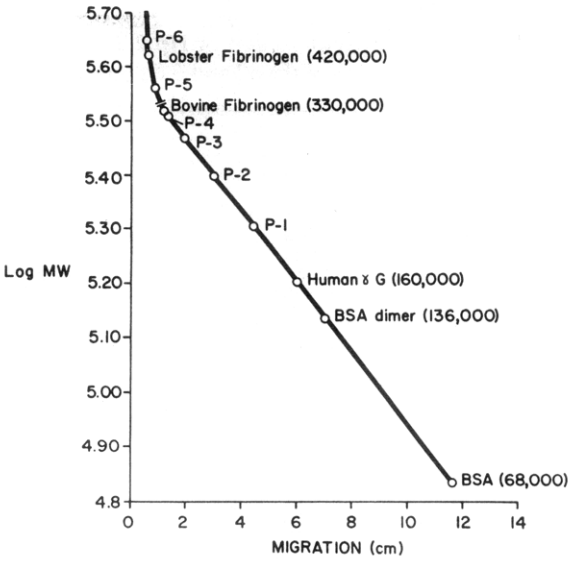

FIGURE 3: Plot of migration distance *vs.* log molecular weight of protein standards and isolated haptoglobin 2–2 polymers.

weight is calculated using the detergent gel technique a value of 110,000 daltons is obtained. Figure 3 shows a plot of migration distance *vs.* the logarithm of the molecular weight of the reference proteins. The migration distance of the haptoglobin 2-2 polymers is plotted on the same graph. Determination of the molecular weight of the haptoglobin polymers by the detergent gel technique indicated that each haptoglobin polymer band differed from the previous band by an average increment of 52,400. The molecular weights of polymers 2–5 were determined using the analytical ultracentrifuge. At least two different determinations were made for each polymer. The molecular weights calculated corresponded closely to both the theoretical and observed molecular weight estimated by the sodium dodecyl sulfate acrylamide procedure, shown in Table II. In Table I the theoretical and observed molecular weight values and polypeptide chain composition of each polymer is given based on the model of $\alpha 2/\beta$ subunit addition.

Chemical Composition of Isolated Haptoglobin Polymers. The complete amino acid sequence of the $\alpha 2$ haptoglobin polypeptide chain has been determined (Black and Dixon, 1968) and the amino acid sequence of a large portion of the haptoglobin β chain has also been established (Barnett *et al.*, 1970, 1972). Amino acid analyses were performed on Hp 2-2 and four of its isolated polymers. The amount of protein analyzed was estimated by use of the extinction coefficient for Hp. Results of triplicate analyses are presented in Table III expressed as residues of amino acid per mole of $\alpha 2/\beta$ subunit (mol wt 57,000). Comparison of these results with the actual number of amino acids known to occur in the $\alpha 2$ plus β polypeptide chains indicates that the $\alpha 2/\beta$ species is the basic sub-

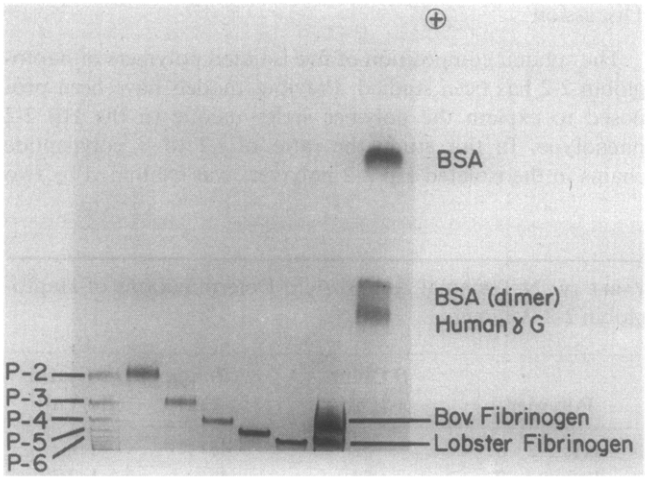

FIGURE 2: Sodium dodecyl sulfate polyacrylamide gel electropherogram of haptoglobin 2–2 polymers, isolated haptoglobin and molecular weight marker proteins.

TABLE I: Molecular Weights of Haptoglobin Polymers.

Polymer Nomenclature	Proposed Model	Theor Mol Wt	Obsd Mol Wt: SDS Electrophoresis[a]	Obsd Mol Wt: Ultracentrifuge
Polymer 1	$\alpha_3\beta_3$	171,000	203,000	
Polymer 2	$\alpha_4\beta_4$	228,000	251,000	224,000
Polymer 3	$\alpha_5\beta_5$	285,000	294,000	291,000
Polymer 4	$\alpha_6\beta_6$	342,000	348,000	340,000
Polymer 5	$\alpha_7\beta_7$	399,000	406,000	399,000
Polymer 6	$\alpha_8\beta_8$	456,000	464,000	

[a] An average of nine different determinations using sodium dodecyl sulfate polyacrylamide gel electrophoresis.

TABLE II: Molecular Weight of Hp Polymer Determined by Sedimentation Equilibrium.

Theor Mol Wt	No. of Determinations	Exptl Mean	Deviation	SE
Polymer 2 (229,000)	3	224,800	18,800	±10,900
Polymer 3 (286,000)	2	291,300	7,500	±5,300
Polymer 4 (344,000)	3	340,400	15,600	±9,000
Polymer 5 (401,000)	2	399,000	22,200	±15,700

TABLE III: Amino Acid Composition of Hp 2-2, α2 Chain, β Chain, and Four Purified Polymers Compared to the Sum of the Amino Acid Compositions of the α2 and β Polypeptide Chains.

Amino Acid[a]	α2	β	Hp 2-2	Polymer 2	Polymer 3	Polymer 4	Polymer 5	α2 + β
Lysine	15	22	37.7	40.1	42.5	32.3	38.9	37
Histidine	4	10	14.3	16.3	19.6	15.0	17.9	14
Arginine	4	5	9.2	10.0	8.5	9.9	8.5	9
Aspartic acid	23	28	50.3	49.2	52.1	50.9	49.6	51
Threonine	5	19	24.9	22.7	23.9	23.4	24.0	24
Serine	3	15	20.7	17.6	19.0	18.8	19.1	18
Glutamic acid	16	29	47.9	45.6	47.1	45.8	36.6	45
Proline	11	13	23.4	25.1	24.9	23.3	23.7	24
Glycine	12	20	31.2	31.6	32.4	31.8	31.7	32
Alanine	8	21	30.5	29.3	27.5	29.1	30.0	29
Half-cystine	6	6	11.3	11.0	8.7	10.4	10.0	12
Valine	12	27	37.3	35.7	37.2	35.6	36.8	39
Methionine[b]	0	5	4.1	3.8	3.8	4.5	3.8	5
Isoleucine	5	13	17.4	17.9	18.0	18.5	18.2	18
Leucine	6	23	29.8	29.4	29.6	29.9	29.9	29
Tyrosine	10	12	19.9	19.6	16.6	17.1	13.7	22
Phenylalanine	0	8	8.2	8.5	8.5	7.9	8.4	8

[a] Results are expressed as residues of amino acid per mol of α2 plus β chain. [b] Five methionine residues are present in the β chain (Barnett *et al.*, 1972) and none in the α2 chain (Black and Dixon, 1968). One residue of methionine is presumed lost during acid hydrolysis.

unit of all polymers examined. When the results of amino acid analysis were expressed assuming any other subunit (for example, 2α2/β or α2/2β) there was no correlation between experimental results and theoretical chain composition. From these analyses it is clear that any model of the Hp 2-2 polymers must be based on the presence of α2 and β polypeptide chains in equal proportion. This information taken together with the molecular weights (Table I) of the individual polymer bands provides direct evidence that each polymeric entity differs from the next member of the series by a subunit composed of an α2 and β polypeptide chain.

Amino-Terminal Analysis. The amino-terminal residues for both the α2 and β polypeptide chains have been determined as valine and isoleucine, respectively (Black and Dixon, 1968; Smith *et al.*, 1962). Table IV shows the ratio of isoleucine to valine for Hp 2-2 and three of the isolated polymers. That these ratios are very close to one in each case reinforces the notion that equal numbers of α2 and β chains occur in each individual polymer.

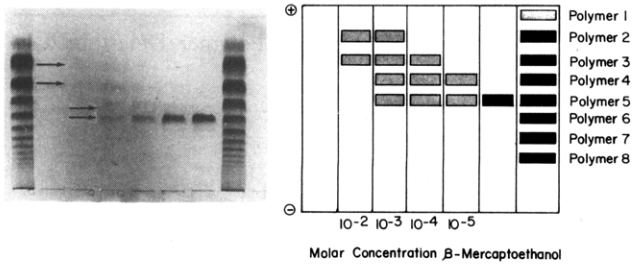

FIGURE 4: Polyacrylamide gel electropherogram of haptoglobin 2–2 and polymer 5 after 6-hr incubation in various concentrations of β-mercaptoethanol. Polymers 2, 3, 4, and 5 are shown by arrows (left). Diagram of banding pattern which appears on the gel is shown on right.

Effect of Variable Concentrations of Reducing Agent on Polymer Conformation. Aliquots of isolated Hp polymers were incubated at 37° for 6 hours in the presence of various concentrations (10^{-5}–10^{-1} M) of β-ME. The effect of low β-ME concentrations on polymer 5 can be seen in Figure 4. Polymer dissociation increases proportionally with increasing concentrations of β-ME. Hp polymer 4 and polymer 5 predominate in 10^{-4} M β-ME. However, at β-ME concentrations of 10^{-3} M, polymer 2–5 are present in approximately equal amounts. When this same experiment was performed in the presence of 0.1% sodium dodecyl sulfate, a new band appeared in the electrophoretic pattern which corresponded to a protein with a molecular weight of approximately 57,000, indicating the release of α2/β subunits from the polymer.

Discussion

The subunit composition of five isolated polymers of haptoglobin 2-2 has been studied. Previous models have been proposed to explain the polymer series unique to the Hp 2-2 phenotype. In this study the ratio of α2 to β polypeptide chains in the isolated Hp 2-2 polymers was examined by two

TABLE IV: N-Terminal Amino Acid Determinations of Haptoglobin 2-2 Polymers.

Polymer	β Chain (% Ile)	α Chain (% Val)	β/α Ratio (Ile/Val)
HP 2-2	52	48	1.08
Polymer 2	54	46	1.17
Polymer 3	54	46	1.17
Polymer 4	55	45	1.22

different methods: quantitative N-terminal analysis and total amino acid analysis. N-Terminal analysis of polymers 2, 3, and 4 (Table IV) revealed that valine and isoleucine occur as N-terminal amino acids in approximately equal amounts in each sample. Hence the $\alpha 2$ and β polypeptide chains are present in a 1:1 ratio in each of the polymers studied. Further information regarding this model was obtained by examining the amino acid composition of polymers 2–5. As shown by the data presented in Table III, the amino acid composition of each of the polymers studied agrees very closely with the theoretical composition of an $\alpha 2/\beta$ subunit. Thus on the basis of amino acid analysis and quantitative N-terminal analysis, any models of polymer composition must include the $\alpha 2$ and β polypeptide chains in equal proportion.

The molecular weights of several isolated polymers were determined. The theoretical consideration of sodium dodecyl sulfate gel electrophoresis has recently been reviewed by Chromback and Rodbard (1971) and Neville (1971). The technique of using sodium dodecyl sulfate polyacrylamide gel electrophoresis for estimating molecular weights of multichain proteins has been seriously questioned by Fish *et al.* (1970). It is interesting that when the molecular weight of Hp 1-1 was determined by the sodium dodecyl sulfate gel technique the molecular weight was within 10% of the known value. When the molecular weights for polymers 2–5 were determined in the analytical ultracentrifuge, the values corresponded quite well with those estimated by the sodium dodecyl sulfate procedure. It appears that the molecular weight of some multimeric proteins can be estimated fairly well by sodium dodecyl sulfate gel electrophoresis. A persistent problem in using this procedure for estimation of molecular weights is the selection of suitable molecular weight marker proteins. The polymers 2, 3, and 4 presented no difficulty because they reproducibly migrated to positions on the linear portion of the standard curve (Figure 3). However it was more difficult to determine accurately the molecular weights of the larger polymers by this method. Utilization of lobster and bovine fibrinogen as reference proteins facilitated the molecular weight estimation of polymer 5. The molecular weights determined by electrophoresis in sodium dodecyl sulfate and by the analytical ultracentrifuge indicated that the molecular weight difference between each successive polymer was approximately 54,500. This information suggested that each successive polymer was formed by the addition of a subunit consisting of an $\alpha 2$ (17,300) and a β (40,000) polypeptide chain.

Other possibilities of polymer composition for Hp 2-2 besides the addition of $\alpha 2/\beta$ subunits were ruled out by the data presented in this study. The suggestion by Marinis and Ott (1965) that each polymer was formed by doubling $(\alpha 2/\beta)_n$, where $n = 2, 4, 8, \ldots$, was not substantiated by the molecular weight estimations presented here. That each successive polymer differs by addition of a β polypeptide chain can also be negated by the quantitative N-terminal and amino acid analysis data. We have explored several other subunit addition models ($2\alpha 2/\beta$, $\alpha 2/2\beta$) and find that only one is consistent with results presented here, namely, polymer compositions of equal amounts of $\alpha 2$ and β chains and an increase in size by the addition of an $\alpha 2/\beta$ subunit. Whether the polymers are formed at the time of synthesis or whether they polymerize later remains to be determined.

It has been shown that after the polymers are isolated and then examined by electrophoresis a small quantity of a heavier molecular weight Hp polymer appears. The minor band migrates to a position that corresponds to a polymer that is a dimer of the main component. For example, preparations of

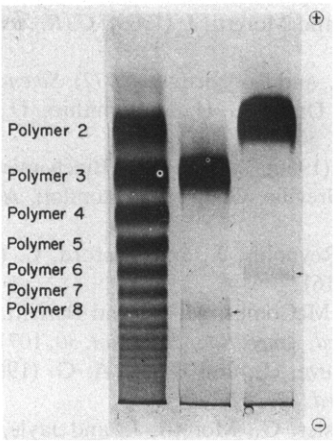

FIGURE 5: Polyacrylamide gel electropherogram demonstrating the presence of dimers of polymers 2 and 3. Dimers are indicated by arrows.

polymer 2 ($\alpha_4\beta_4$, mol wt 232,000) exhibit a minor band which corresponds to polymer 6 ($\alpha_8\beta_8$, mol wt 464,000). Figure 5 shows the electrophoretic pattern of the major and minor bands of the isolated haptoglobin polymers. This observation could suggest that after a certain polymer size is reached the other higher molecular weight species are formed *de novo* by simple dimerization. It could also mean, however, that once the polymers have been isolated and concentrated the dimers formed are simply due to artifacts of purification. Since the amount of dimer formed is considerably less than that seen in unfractionated Hp, our reasoning is that the latter instance is more probable.

It is known that the α and β polypeptide chains comprising the haptoglobin molecule are linked through disulfide bridges (Connell *et al.*, 1962). These experiments demonstrate that the haptoglobin polymers are also linked through disulfide bonds. The dissociation of an isolated polymer into the next lighter member of the series by exposure to low concentrations of β-ME indicates that disulfide bridges are important in maintaining the integrity of each polymer. The fact that mild reduction leads to the lighter polymeric forms indicates that the disulfides holding the $\alpha 2/\beta$ subunits together to form the polymers are more labile than the disulfides linking the $\alpha 2$ to the β chains. This suggests that the disulfide linkage between the $\alpha 2$ and β chain is located on the interior portion of the molecule whereas the disulfide linkage between polymer subunits is near the surface and therefore more accessible to reduction.

Acknowledgments

We acknowledge Mr. J. Mark Boughter for superior technical assistance. Dr. Eldon Sutton of the University of Texas at Austin kindly provided the electrophoretic elution apparatus.

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Mössbauer Studies of Cytochrome P-450$_{cam}$†

M. Sharrock,* E. Münck, P. G. Debrunner, V. Marshall, J. D. Lipscomb, and I. C. Gunsalus

ABSTRACT: The heme protein cytochrome P-450$_{cam}$ from *Pseudomonas putida* was investigated by Mössbauer spectroscopy in both oxidized and reduced states. The oxidized enzyme in the presence of the substrate, camphor, contains a mixture of high-spin ($S = 5/2$) and low-spin ($S = 1/2$) ferric heme sites. The high-spin fraction increases as temperature is raised. Removal of camphor results in a conversion from high spin to low spin. Hyperfine parameters that approximately describe the experimental spectra were calculated. Anaerobic reduction of P-450$_{cam}$ in camphor solution produces a high-spin ferrous ($S = 2$) state. Exposure of this preparation to oxygen results in a new complex whose Mössbauer spectra are similar to those observed for oxygenated hemoglobin. Both proteins show large quadrupole splitting and only moderate isomer shift relative to iron metal; no paramagnetic effects are observed even in large applied magnetic fields. Such spectra appear to be characteristic of the heme group with an O_2 molecule as one axial ligand. P-450$_{cam}$ also forms a stable adduct with carbon monoxide. The Mössbauer spectra of this complex are very similar to those of hemoglobin carbon monoxide.

Cytochromes of the P-450 type are identified by a Soret band near 450 nm when reduced anaerobically to the ferrous form and complexed with carbon monoxide. These proteins are found in many organisms, where they have various metabolic functions. In mammals, these include fatty acid oxidation, steroid hydroxylation, and drug detoxification. Gunsalus and coworkers (Hedegaard and Gunsalus, 1965; Katagiri *et al.*, 1968) have described the role of a bacterial cytochrome P-450 (designated P-450$_{cam}$) in the methylene hydroxylation of D(+)-camphor in *Pseudomonas putida*. This enzyme, the subject of our study, has a molecular weight of 45,000 and is composed of a single polypeptide chain, one ferriprotoporphyrin IX group, and a small carbohydrate unit (Tsai *et al.*, 1971). A proposed reaction mechanism, involving two other proteins, is shown in Figure 1 (Gunsalus *et al.*, 1971; Estabrook *et al.*, 1971). All three enyzmes are present in the soluble fraction of the bacterial cells, providing an easily accessible, well-defined hydroxylase system. Knowledge gained in this work will be a step toward understanding the operation of analogous membrane-bound systems found in the mitochondria and microsomes of higher life forms.

The proposed reaction mechanism involves transfer of reducing equivalents to P-450$_{cam}$ from reduced nicotinamide adenine dinucleotide (NADH) *via* a flavoprotein and an iron–sulfur protein (Gunsalus *et al.*, 1971). The catalytic process

† From the Department of Physics and Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801, *Received August 24, 1972.* Supported in part by grants from the U. S. Public Health Service (GM 16406 and AM 00562).