Functional and Structural Properties of *Murex fulvescens* Hemocyanin: Isolation of Two Different Subunits Required for Reassociation of a Molluscan Hemocyanin†

Marius Brouwer,*,‡ Margaret Ryan, Joseph Bonaventura,§ and Celia Bonaventura

ABSTRACT: The hemocyanin of the gastropod mollusc *Murex fulvescens* is present in the hemolymph as a high molecular weight aggregate which has a sedimentation coefficient of 100 S. It can be dissociated into 11S subunits which are uncommonly large polypeptide chains. This is typical of molluscan hemocyanin subunits which are considered to be made up of 7 to 8 oxygen binding domains of about 50 000 daltons each. Experiments with *Murex* hemocyanin provide the first direct evidence of subunit diversity in a molluscan hemocyanin. The subunits of *Murex fulvescens* hemocyanin can be fractionated into two components which will be called subunits A and B, respectively. They migrate as single bands on regular disc gels. The native 100S molecule is composed of approximately equimolar quantities of these subunits and can thus be written as $A_{10}B_{10}$. Both subunits A and B are required for reassembly of the 100S molecule from its 11S subunits. The reassociated hemocyanin exhibits cooperative interactions and pH dependence of oxygen binding equilibria and kinetics which are very similar to those of the undissociated molecules found in the hemolymph. Amino acid composition, neutral hexose content, and functional behavior of subunits A and B are very similar. The equilibria and kinetics of oxygen binding by both subunits are heterogeneous. Heterogeneity in this hemocyanin is therefore not only found on the subunit level, but on the domain level as well.

Oxygen carrying proteins fall into three major classes: hemoglobins, hemerythrins, and hemocyanins. The blue-colored hemocyanins are the respiratory pigments which occur in the hemolymph of many molluscs and arthropods. They constitute a class of high molecular weight oligomers. All of them have dimeric copper centers to which one oxygen molecule can be bound. Hemocyanins have been studied as elaborate systems of self-assembly, as models of cooperative and allosteric interactions, and as examples of protein–metal complexes (Van Holde & Van Bruggen, 1971; Lontie & Witters, 1973; Antonini & Chiancone, 1977; Hendrickson, 1977; Bonaventura et al., 1977a). Arthropod hemocyanins occur as oligomers containing 6, 12, 24, or 48 oxygen binding subunits, each having a molecular weight of about 75 000. It is now fairly well established that subunit diversity occurs in a large number of arthropodan hemocyanins (Sullivan et al., 1974, 1976; Bonaventura et al., 1975; Sugita & Sekiguchi, 1975; Murray & Jeffrey, 1974; Jeffrey et al., 1976; CARPENTER & Van Holde, 1973; Kuiper, 1976; Lamy et al., 1977; Schneider et al., 1977; Miller et al., 1977).

The subunits of molluscan hemocyanins are very complex structures. They have a molecular weight of about 360 000. This corresponds to 1/20 of the molecular weight of the undissociated 100S molecule (Siezen & Van Bruggen, 1974; Brouwer et al., 1976). Electron micrographs of the subunits show a "string of beads" structure with 7–8 flexibly connected globules (Siezen & Van Bruggen, 1974). This enormous polypeptide chain can be cleaved by limited proteolytic digestion into functional units of about 50 000 molecular weight, or multiples thereof, each containing one oxygen binding site (Brouwer & Kuiper, 1973; Lontie et al., 1973; Bannister et al., 1975; Brouwer et al., 1976, Gielens et al., 1975, 1977; Bonaventura et al., 1977a,b). Subunits of *Helix pomatia* α-hemocyanin seem to be heterogeneous in electrophoretic mobility as judged by the reversible boundary spreading test (Siezen & Van Driel, 1973). The presence of two different polypeptide chains in this hemocyanin was suggested, based upon the interpretation of the time course of the proteolytic digestion of the native molecule (Brouwer, 1975). However, until now, there has been no conclusive evidence regarding the existence of more than one type of subunit in molluscan hemocyanins.

In this paper we present direct evidence for the occurrence of two different subunits in hemocyanin of the mollusc *Murex fulvescens*. We report on their oxygen binding properties and the fact that both subunits appear to be required for reassembly of the 100S molecule.

Methods

Specimens of *Murex fulvescens* were collected offshore in the vicinity of Beaufort, N.C. Animals were bled by removing the apex of the shell and allowing the hemolymph to drip into a beaker containing a few milligrams of the protease inhibitors sodium tetrathionate and α-toluenesulfonyl fluoride (Waxman, 1975) and EDTA[1] (Gielens et al., 1975). The hemolymph was first centrifuged for 20 min at 12 062*g* to remove particulate matter and then in a Beckman Model L preparative ultracentrifuge for 75 min at 144 000*g*. The pellets were dissolved in 50 mM Tris (pH 7), ionic strength 0.1 containing 10 mM $CaCl_2$ and chromatographed on a 4 × 50 cm column of Sepharose CL-4B in the same buffer. The hemocyanin concentration was approximated from the absorbance at 345 nm using an $E_{cm}^{1\%} = 3.28$ as determined for *Busycon canaliculatum* hemocyanin (Nickerson & Van Holde, 1971). *Murex*

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[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DEAE, diethylaminoethane; p_{50}, partial oxygen pressure at half-saturation.

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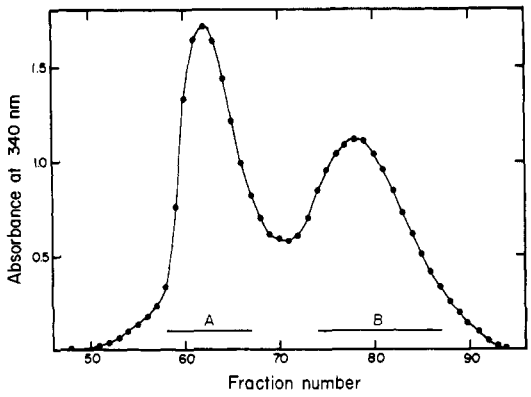

FIGURE 1: Ion-exchange chromatography of dissociated *Murex fulvescens* hemocyanin in 50 mM Tris, 10 mM EDTA (pH 8.9) at room temperature on a 20 × 2.5 cm column of DEAE-Sepharose CL-6B. Gradient was 0.2–0.6 M NaCl. Flow rate was 40 mL per h. Fractions of 4 mL were collected and read at 340 nm (●).

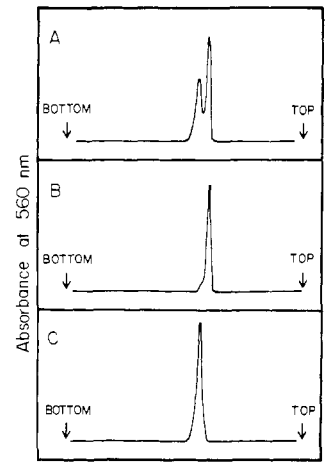

FIGURE 2: Spectrophotometric scan at 560 nm of the electrophoretic pattern of dissociated *Murex fulvescens* hemocyanin in 50 mM Tris, 10 mM EDTA (pH 8.9) on 4% polyacrylamide gels. (A) Dissociated whole hemocyanin. (B) Subunits A. (C) Subunit B. Subunits A and B isolated as shown in Figure 1.

hemocyanin was dissociated into its subunits by dialysis against 50 mM Tris, 10 mM EDTA (pH 8.9). Hemocyanin subunits were isolated by ion-exchange chromatography on 2.5 × 20 cm DEAE Sepharose CL-6B (50 mM Tris, 10 mM EDTA (pH 8.9) with a 0.2–0.6 M linear gradient of NaCl).

Polyacrylamide disc gel electrophoresis was carried out using a gel concentration of 4%, with 2.5% cross-linking. Buffers used for ion exchange chromatography and electrophoresis were the same: 50 mM Tris (pH 8.9) containing 10 mM EDTA. The electrophoresis buffer was changed after 2 h of electrophoresis in order to prevent the formation of a pH gradient between the anode and cathode. Total electrophoresis time was 4 hours. Sodium dodecyl sulfate gel electrophoresis on 4% polyacrylamide gels with 2.5% cross-linking was carried out as described previously (Brouwer & Kuiper, 1973). Hemocyanin was collected as described above and samples were immediately denatured either at room temperature or at 100 °C in the following buffers: 50 mM Tris pH 8.5 containing 10 M urea, 2% sodium dodecyl sulfate, and 2% mercaptoethanol or 6 M guanidine hydrochloride plus 2% mercaptoethanol; 0.1 M pyridine-formate (pH 2.8) containing 10 M urea or 6 M guanidine hydrochloride. Prior to electrophoresis the samples were extensively dialyzed against Tris, ionic strength 0.01, pH 7.5, containing 0.1% sodium dodecyl sulfate. Total hexose content was determined with the phenol-sulfuric acid method (Dubois et al., 1956), with glucose as a standard. Amino acid analyses were done on a Kontron Model Liquimat III analyzer. Hydrolysates were measured after 24, 48, and 72 h of hydrolysis in vacuo in 6 N HCl at 110 °C. Cysteine was determined as cysteic acid following oxidation by performic acid. Sedimentation velocity, oxygen binding, and stopped-flow spectrophotometry were performed as described previously (Brouwer et al., 1977).

Results

Murex hemocyanin, purified by preparative ultracentrifugation, elutes as a single peak from columns of Sepharose CL-4B. It sediments as a single component having a sedimentation coefficient of 100 S, a value characteristic of gastropod hemocyanins (Van Holde & Van Bruggen, 1971). After complete dissociation of the 100S structure by dialysis against 50 mM Tris, 10 mM EDTA, pH 8.9, the *s* value is reduced to approximately 11. The elution profile obtained after ion-exchange chromatography of the 11S subunits is shown in Figure 1. Integration of the peaks from columns like those shown in Figure 1 shows that roughly equal quantities of the subunits

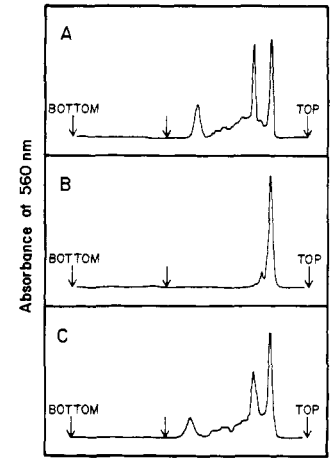

FIGURE 3: Spectrophotometric scan at 560 nm of the electrophoretic pattern of sodium dodecyl sulfate denatured *Murex fulvescens* hemocyanin on 4% polyacrylamide gels. (A) Subunit B. (B) Subunit A. (C) Whole hemocyanin. Subunits A and B were isolated as shown in Figure 1. The arrows indicate the position of transferrin, molecular weight 77 000.

are present. Rechromatography of each of the separated subunits gave a single peak coinciding with the elution volume expected on the basis of the initial separation. Hereafter, we refer to the peaks as subunits A and B. Sedimentation analysis of subunits A and B in 50 mM Tris, 10 mM EDTA, pH 8.9, showed that both subunits correspond to a single 11S boundary.

Disc gel electrophoresis of the unseparated 11S subunits shows two bands (Figure 2A). Subunit A corresponds to the slower moving band (Figure 2B) and subunit B to the faster one (Figure 2C). Sodium dodecyl sulfate electrophoresis shows a more complex pattern. Despite the use of protease inhibitors, three major molecular weight species are present in the denatured 100S molecule (Figure 3C), irrespective of the various methods of denaturation (see Methods). Subunit A corresponds to the highest molecular weight species (Figure 3B). Subunit B is composed of the high molecular weight species and two lower molecular weight species (Figure 3A).

The Hill plots of oxygen binding of subunits A and B are straight lines. Their slopes and standard deviations were calculated by linear regression analysis of the 12 data points for each binding curve. The Hill plot of subunit A has a slope of

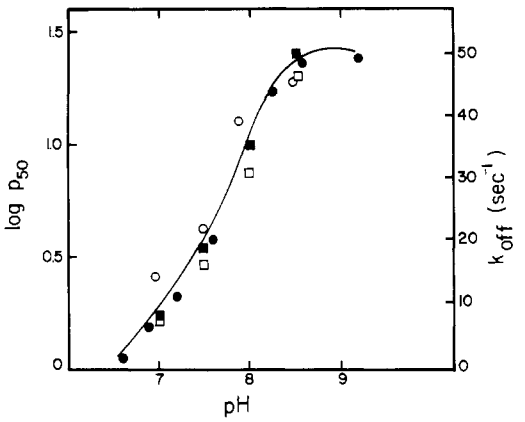

FIGURE 4: Effect of the pH on the oxygen affinity and the apparent oxygen dissociation rate of undissociated and reassociated *Murex fulvescens* hemocyanin. k_{off} was calculated from the slope of the first-order plots between 50 and 20% of the deoxygenation reaction. Hemocyanin, about 4 mg/mL, was in 50 mM Tris buffers of ionic strength 0.1, pH 7–9, to which 10 mM $CaCl_2$ was added. Temperature: 20 °C. (●) p_{50} of undissociated hemocyanin. (○) p_{50} of reassociated hemocyanin. (■) k_{off} of undissociated hemocyanin. (□) k_{off} of reassociated hemocyanin.

TABLE I: Amino Acid Composition in Mole % and Carbohydrate Content in Weight % of Subunits A and B of *Murex fulvescens* Hemocyanin.

	subunit A	subunit B
Asp	11.5	12.1
Thr	5.6	5.1
Ser	5.7	6.2
Glu	11.4	11.6
Pro	5.8	5.7
Gly	6.7	6.6
Ala	6.7	6.8
Val	5.3	5.3
½-cystine	1.5	2.0
Met	1.9	1.8
Ile	3.8	3.4
Leu	9.4	8.6
Tyr	4.6	4.4
Phe	6.5	6.2
Lys	4.3	3.8
His	6.6	7.6
Arg	4.6	4.4
carbohydrate	5.6	5.5

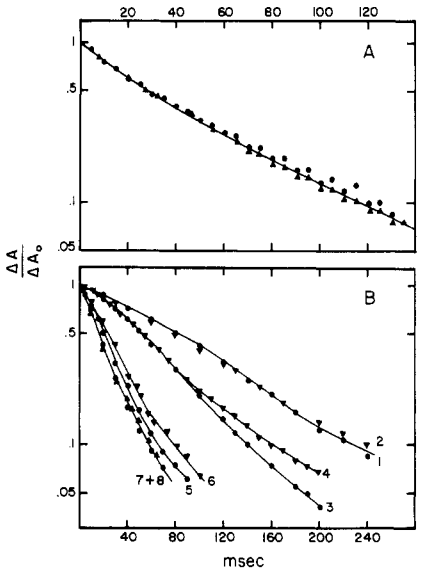

FIGURE 5: (A) Time course of the deoxygenation reaction of subunits A and B in 50 mM Tris, 10 mM EDTA (pH 8.9). (●) Subunit A; (▲) subunit B. (B) Time course of the deoxygenation reaction of undissociated and reassociated oxyhemocyanin in 50 mM Tris, ionic strength 0.1, pH 7–8.5, plus 10 mM $CaCl_2$. ●: (1, 3, 5, and 7) undissociated hemocyanin at pH 7.0, 7.5, 8.0, and 8.5, respectively. ▼: (2, 4, 6, and 8) reassociated hemocyanin at pH 7.0, 7.5, 8.0, 8.5, respectively. Temperature: 20 °C.

0.95 ± 0.01 and a p_{50} value of 3.1 mm of mercury. The Hill coefficient of subunit B is 0.93 ± 0.01 and the p_{50} value is 3.4 mm of mercury. A value of the Hill coefficient which is statistically less than one can be taken as good evidence that there is nonequivalence in the affinity of the oxygen binding sites in solution. Heterogeneity is also manifest in kinetic experiments. Figure 5A shows that the rate of oxygen dissociation from either subunit A or B becomes slower as the reaction proceeds. The fast part of the dissociation reaction has an apparent first-order rate constant of 60 s^{-1} and constitutes about 30% of the reaction. The remaining 70% has an apparent rate constant of 16 s^{-1}.

The role of subunits A and B in the reassembly of the 100S molecule was investigated. Assembly of this structure was accomplished by dialyzing an equimolar mixture of subunits A and B for 48 h at 4 °C vs. 50 mM Tris, ionic strength 0.1, pH 7, in the presence of 10 mM $CaCl_2$. Comparable attempts to reassociate subunit A in the absence of subunit B or vice versa resulted mainly in formation of an aggregate with a sedimentation coefficient of 23S and some larger aggregates. It should be emphasized that no 100S molecules were detected when isolated subunits were studied. The mixture of subunits A and B reassociated into 100S molecules with an efficiency of 50–75%. The remainder had sedimentation coefficients of 23S and 130S. The 100S and 130S (1.5 molecules) material was separated from the 23S component by preparative ultracentrifugation. The mixture containing 100S and 130S molecules was used for gel electrophoresis after dissociation, for the measurement of oxygen binding curves and in rapid mixing experiments. The ratio of subunits A and B in the reassociated molecules was the same as in the native molecules.

Both native 100S and the reassembled mixture of 100S plus 130S show cooperative oxygen binding behavior and a strong negative Bohr effect (Figure 4). Maximum cooperativity occurs at pH 8.5, where the Hill coefficient of the native and reassociated molecules is equal to 4.6 and 3.5 respectively. The kinetics of the process of oxygen dissociation from undissociated and reassembled 100S plus 130S *Murex* hemocyanin were followed by mixing the oxy protein with buffer containing sodium dithionite. The time course of oxygen dissociation at different pH values is shown in Figure 5B. The dissociation process is clearly autocatalytic; that is, the dissociation rate constant increases as the reaction proceeds. The final stages of the process are progressively slower, in accordance with the heterogeneity in oxygen binding behavior of the subunits. The effect of pH on the overall rate of oxygen dissociation is shown in Figure 4. Since the rate of oxygen dissociation changes with the extent of the reaction, we arbitrarily chose to plot the apparent rates as calculated from the slope of the first-order plots between 50 and 20% of the reaction vs. the pH. Figure 4 shows that the oxygen dissociation rate increases with increasing pH.

Carbohydrate content and amino acid composition of subunits A and B are very similar (Table I). The values reported in this table are typical of gastropod hemocyanins (Dijk, 1971).

Discussion

Subunit diversity has now been established in the hemocyanin systems of many arthropods. This diversity is manifest both functionally and structurally (Bonaventura et al., 1977a). The obvious analogy between arthropodan and molluscan hemocyanins leads to the question of possible subunit diversity in molluscan hemocyanins. Previously, only indirect evidence regarding this question has been found. As mentioned in the introductory section, the subunits of *Helix pomatia* α-hemocyanin seem to be heterogeneous in electrophoretic behavior as judged by the reversible boundary spreading test (Siezen & van Driel, 1973). Study of the time course of proteolytic digestion of *Helix pomatia* α-hemocyanin led to the hypothesis that two different polypeptide chains are present in this hemocyanin (Brouwer, 1975). In the present paper we have given direct evidence for the occurrence of multiple subunits in hemocyanin of the mollusc *Murex fulvescens*.

Murex fulvescens hemocyanin can be dissociated into 11 S subunits, the sedimentation coefficient of which shows a normal concentration dependence. The same *s* value has been reported for the dissociation products of *Murex trunculus* hemocyanin (Wood & Peacocke, 1973). According to Siezen & Van Bruggen (1974), the 11S dissociation product of *Helix pomatia* α-hemocyanin corresponds to a 1/20 molecule with a molecular weight of 360 000. This subunit cannot be dissociated into smaller structures and is therefore thought to be the size of the minimal polypeptide chain in gastropod hemocyanins. This chain is folded into 7–8 structural domains of about 50 000 daltons, each containing one oxygen binding site (Brouwer et al., 1976; Siezen & Van Bruggen, 1974). A similar model has been proposed for the polypeptide chain of *Helix pomatia* β-hemocyanin (Gielens et al., 1977). The 1/20 molecules of *Murex fulvescens* hemocyanin could be fractionated into two subunits (Figure 1), present in approximately equimolar amounts. Rechromatography of the separated components emphasized that we were dealing with a noninteracting system. Moreover, disc gel electrophoresis revealed both components to behave as electrophoretically pure species (Figure 2). Subunit a was also pure as judged by sodium dodecyl sulfate gel electrophoresis (Figure 3B) where a single high molecular weight band was observed. Subunit B contained the same high molecular weight band, together with two lower molecular weight species (Figure 3A). These three molecular weight species, when present in the native molecule, chromatographed as a single peak, ran as a single band on disc gels, and sedimented as a single peak with the same sedimentation coefficient as the pure high molecular weight subunit A. This strongly suggests that the two lower molecular weight bands in subunit B have arisen by a single proteolytic cleavage in the polypeptide chain of subunit B, without changing the aggregation state of the subunit. This is supported by the observation that the amount of the three molecular weight species in subunit B varies slightly from one preparation to another. An estimate of 107 000 for the lowest molecular weight species and a minimum molecular weight of 200 000 for the other species is consistent with the proposed proteolytic cleavage. The sum of the sodium dodecyl sulfate gel patterns of subunits A and B equals the sodium dodecyl sulfate gel pattern obtained after denaturation of the 100S molecule (Figure 3). This means that no additional proteolysis takes place during the isolation procedure of the subunits. Therefore proteolysis occurs either in the hemolymph or immediately after the collecting of the hemocyanin. The results described here seem to agree with the situation as it exists in another molluscan hemocyanin. The time course of the proteolytic digestion of *Helix pomatia* α-hemocyanin could be simulated with a model which required the presence of two different polypeptide chains, occurring in equimolar amounts. One chain contained a peptide bond which was extremely susceptible to proteolysis (Brouwer, 1975).

It might be asked if our results are due to heterogeneity or polymorphism in the hemocyanins of several individual *Murex*. This possibility was ruled out by repeating our experiments with the hemocyanin collected from a single *Murex*. The results obtained were identical with those obtained with pooled hemocyanin samples. This indicates that the observed subunit diversity represents heterogeneity within the subunits of a particular individual.

Functional differences between proteolytic fragments isolated from *Octopus* and *Busycon* hemocyanin have been reported (Bonaventura et al., 1977a,b). Circular dichroic spectra of proteolytic fragments isolated from *Helix pomatia* β-hemocyanin indicated the presence of two classes of copper groups (Gielens et al., 1977). These data clearly show that there are differences between the 50 000 dalton structural domains in molluscan hemocyanins. However, it is possible that the differences observed are due to the existence of different polypeptide chains, each of which is composed of functionally identical domains. The pure subunit A from *Murex fulvescens* hemocyanin shows heterogeneous binding behavior and biphasic kinetics of the oxygen dissociation rate (Figure 5A). In the oxygen dissociation process 30% of the reaction has an apparent first-order rate constant of 60 s^{-1}. The remaining 70% has an apparent first-order rate constant of 16 s^{-1}. Since the off-rate seems to be the dominant parameter in determining the stability of the hemocyanin–oxygen complex (see later), we assume that the k_{on} of the two kinetic species are similar. In this case the equation describing the oxygen saturation of the subunits as a function of the pO_2 becomes:

$$Y_t = \frac{K_1 p}{1 + K_1 p} 0.7 + \frac{K_2 p}{1 + K_2 p} 0.3$$

in which $p = pO_2$, K_1 and K_2 are the equilibrium association constants, and $K_1 = 4K_2$. The Hill plot has been calculated, and linear regression shows the slope to have a value of 0.936 ± 0.003, in complete accordance with the experimental values of 0.95 and 0.93 for subunits A and B, respectively. The data mentioned above show that the oxygen binding sites in subunit A are nonequivalent, indicating that the domains in a single pure polypeptide chain are functionally different from each other.

The reassociation procedure as described in this paper led to the finding that both subunits A and B are required for the reassembly of the 100S molecule which can then be written as $A_{10}B_{10}$. Subunit A reassociates mainly into a 23S aggregate. Subunit B behaves similarly. When the 23S aggregates of subunits A and B are combined and dialyzed vs. reassociation buffer, no further association is observed, indicating that the 23S material is a stable, but misfit aggregate. When subunits A and B are combined and dialyzed vs. the reassociation buffer, approximately 50–75% of the hemocyanin reassembles into 100S and 130S molecules. Based on the behavior of the isolated subunits in self-assembly experiments, it is not surprising that some 23S aggregate is formed. These results show that the two types of 11S subunits occur in the same 100S molecule. Our reassembly studies agree with those of Siezen & Van Bruggen (1974) who reported incomplete reassociation of *Helix pomatia* α-hemocyanin subunits into the 100S molecule. However, *Murex trunculus* hemocyanin could not be reassociated from its subunits either by lowering the pH, or by the addition of magnesium ions or both (Wood & Peacocke, 1973). It is relevant to the reassembly experiments described here for *Murex* hemocyanin that the arthropodan subunits also play

a distinct role in the assembly process. Hemocyanin of the horseshoe crab *Limulus polyphemus* can be fractionated into at least five chromatographic zones (Sullivan et al., 1974). Recent experiments have shown that zones II and III of *Limulus* hemocyanin can reassemble into hexamers. Zone V participates in the hexamer to dodecamer reassociation. Zone IV seems to be involved in the production of 24-mers (Bonaventura et al., 1977a; Schutter et al., 1977). The requirement of multiple polypeptides in the assembly of large functional aggregates has also been observed in the hemocyanin systems of the fresh water crayfish *Cherax destructor* (Murray & Jeffreys, 1974; Jeffreys et al., 1976) and the scorpion *Androctonus australis garzonii* (Lamy et al., 1977).

The oxygen binding and oxygen dissociation rate experiments, as a function of pH, show that the functional properties of undissociated and reassociated 100S molecules are very similar (Figure 4). Increasing the pH values lowers the oxygen affinity, giving rise to a marked negative Bohr effect. Kinetically, the decrease in affinity at high pH is correlated with an increase of the oxygen dissociation rate (Figure 4). The observation that the stability of the oxygen-hemocyanin complex is largely determined by the dissociation constant has also been described for *Limulus* (Brouwer et al., 1977) and *Panulirus* hemocyanin (Kuiper et al., 1975, 1978). The time course of oxygen dissociation is autocatalytic, which is quite common among respiratory proteins, both hemocyanins and hemoglobins (Antonini & Brunori, 1971; van Driel et al., 1974; Sullivan et al., 1974; Bonaventura et al., 1975; Brouwer et al., 1977; Kuiper et al., 1978). This autocatalytic tendency is generally interpreted as being a kinetic reflection of cooperativity. The decrease of the final rates of oxygen dissociation is probably a reflection of the nonequivalency of the binding sites of the various domains. The initial slope of the first order plots at pH 7 and 7.5 (Figure 5B) corresponds to an apparent oxygen dissociation rate of 10 s^{-1}. This is very similar to the value reported for the oxygen dissociation rates of the high affinity state of *Limulus* hemocyanin (Brouwer et al., 1977), *Penaeus* hemocyanin (Brouwer et al., 1978), and *Helix pomatia* hemocyanin (van Driel et al., 1974), 8, 10, and 10 s^{-1}, respectively, showing that the high affinity state of these hemocyanins is characterized by the same slow rate of oxygen dissociation. Faster dissociation rates are observed for the high affinity states of *Panulirus interruptus* hemocyanin (Kuiper et al., 1978) and *Buccinum undatum* hemocyanin (Wood et al., 1977), 35 and 80 s^{-1}, respectively.

The results presented here show that *Murex fulvescens* hemocyanin contains two different types of subunits, which are functionally very similar, but structurally different. The domains within these large subunits seem to be functionally different. Heterogeneity is thus found not only on the subunit level but on the domain level as well (Bonaventura et al., 1977a,b). The understanding of the role of the different subunits and domains in the functional behavior and self-assembly of this huge molecule is an intriguing problem that is presently the subject of further study.

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Unique Human Glycoprotein, α_1-Microglycoprotein: Isolation from the Urine of a Cancer Patient and Its Characterization†

Ben K. Seon* and David Pressman

ABSTRACT: A human glycoprotein was isolated from the urine of a patient with plasma cell leukemia. It appears pure and homogeneous when examined by immunoelectrophoresis, sodium dodecyl sulfate ($NaDodSO_4$)-polyacrylamide gel electrophoresis, gel filtration in 6 M guanidine hydrochloride (Gdn·HCl), and NH_2-terminal amino acid sequence analysis. It has a brown color due to a tightly (most likely covalently) bound chromophore group(s) and migrates to the α_1 region in immunoelectrophoresis. A molecular weight (mol wt) of 27 000 was obtained for the glycoprotein by gel filtration in 6 M Gdn·HCl. Its approximate mol wt determined by $NaDodSO_4$-polyacrylamide gel electrophoresis is 29 000 on 5% and 7.5% and 10% gels. Amino acid and hexosamine analyses showed that it is a glycoprotein and indicated that it contains four half-cystine residues per molecule. Based on the above observations we designated it "α_1-microglycoprotein" (α_1-MGP). Isoelectric focusing of α_1-MGP showed a significant charge heterogeneity, although only a single NH_2-terminal amino acid sequence was obtained for α_1-MGP, i.e., Gly-Pro-Val-Pro-( )-Pro-Pro-Asx-Asx-Ile-Glx-Val-Glx-Glx-Asx-Phe-Phe-Ile-(Ser or Ala)-Arg. The α_1-MGP was found in significant concentrations in the urine of many patients with neoplastic diseases.

We have previously reported the results of our study of several immunoglobulin-related proteins in the urine of a patient TSCH with plasma cell leukemia (Seon et al., 1977a; Seon & Pressman, 1977). The amounts of these proteins in the patient paralleled the clinical manifestations during the course of illness (Gailani et al., 1977a).

In the present study we have isolated a unique glycoprotein, α_1-microglycoprotein,[1] from the urine of the same patient TSCH by a relatively simple procedure and have characterized its chemical, physicochemical, and immunological properties. Recently several groups reported similar proteins, although there are some discrepancies in the properties of these proteins (Tejler & Grubb, 1976; Frangione et al., 1976; Svensson & Ravnskov, 1976; Ekström & Berggård, 1977). None of these reported proteins were obtained from cancer patients and the procedures used for their isolation were significantly different from that used here.

Experimental Section

Materials

Urine. The urine specimens were collected from a single patient TSCH with relapsed plasma cell leukemia during 24-h periods. During the collection, NaN_3 was always present at concentrations higher than 0.1%.

Antisera. Rabbit or goat antisera specific to each of the immunoglobulin component chains, i.e., κ, λ, γ, α, μ, δ and ϵ chains, were prepared and characterized in our laboratory (Seon & Pressman, 1974; Seon et al., 1977a). The antiserum specific to β_2m[2] (Seon & Pressman, 1977) and antisera to normal human serum and normal human urine were also prepared and characterized in our laboratory. Antisera specific to carcinoembryonic antigen and urinary kallikrein were kindly provided by Dr. S. Harvey of Roswell Park Memorial Institute and Dr. O. Ole-MoiYoi of Harvard Medical School, respectively. The antisera specific to the following human serum components were purchased from Behring Diagnostics (through American Hoechst Corp., N.J.): α_1-acid glycoprotein, albumin, α_1-antichymotrypsin, α_1-antitrypsin, antithrombin III, C-reactive protein, C1q component, C1s component, C1s inhibitor, C3/C3c, C3 activator, ceruloplasmin,

† From the Department of Immunology Research, Roswell Park Memorial Institute, Buffalo, New York 14263. *Received February 7, 1978.* Roswell Park Memorial Institute is a unit of the New York State Department of Health. This study was supported in part by Grants CA 17609 and CA19304 from the National Cancer Institute, Department of Health, Education and Welfare.

[1] The glycoprotein we purified was designated as α_1-microglycoprotein since it migrates to the α_1 region in immunoelectrophoresis and is a small glycoprotein with molecular weight of 27 000.

[2] Abbreviations used: α_1-MGP, α_1-microglycoprotein; β_2m, β_2-microglobulin; Dnp, dinitrophenyl; $NaDodSO_4$, sodium dodecyl sulfate; Gdn·HCl, guanidine hydrochloride; mol wt, molecular weight.

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