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Oxygen-Linked Association–Dissociation of *Helix pomatia* Hemocyanin†

Roel van Driel* and Ernst F. J. van Bruggen

ABSTRACT: Hemocyanin of *Helix pomatia* (molecular weight 9×10^6) contains approximately 180 oxygen-binding sites. Oxygen-binding and oxygen-linked association-dissociation phenomena of *Helix pomatia* α-hemocyanin at high ionic strength (I = 1.1) (pH 8.2) have been investigated. Oxygen binding is cooperative. Under oxygen-free conditions 65% of the protein is present as half molecules (molecular weight 4.5×10^6) and 35% as one-tenth molecules (molecular weight 0.9×10^6). Upon oxygen binding an abrupt, complete dissociation of the half molecules into one-tenth molecules is observed. Evidence is presented that both the associated and the dissociated states bind oxygen noncooperatively, the observed cooperativity being almost completely due to the ligand-linked dissociation. The oxygen pressures at half-saturation of the dissociated and associated proteins are approximately 4 and 40 mm, respectively. These oxygen-binding properties are compared to those under more physiological conditions. It is suggested that formation of a closed ring of five one-tenth subunits (*i.e.* association of subunits to a half molecule) is a necessary condition to constrain the protein to a state with low oxygen affinity.

The structure and oxygen-binding properties of α-hemocyanin of the Roman snail, *Helix pomatia,* and other molluscs have been studied extensively in the past few years (Van Holde and Van Bruggen, 1971; Er-el *et al.,* 1972; Mellema and Klug, 1972; Brouwer and Kuiper, 1973; Van Driel, 1973; Siezen, 1973). *Helix pomatia* hemocyanin has a molecular weight of 9×10^6, and contains about 180 oxygen binding sites. Under proper conditions of pH, ionic strength, and divalent cation concentration, half, one-tenth, and one-twentieth molecules can be observed (Siezen, 1973).

Siezen (1973) presented evidence that one-tenth subunits, under liganded conditions, can exist in two grossly different conformations. At low ionic strength (I = 0.1) the structure is rather loose, whereas at high salt concentrations (I = 1) the subunit is more compact. At low ionic strength, pH 8.2, one-tenth subunits bind oxygen noncooperatively. This paper reports the oxygen-binding properties of hemocyanin at high ionic strength, pH 8.2. Under these conditions oxygen binding is cooperative and is linked to a monomer-pentamer equilibrium between one-tenth and half molecules. The oxygen affinity of the pentamer is about tenfold lower than that of the monomer, and is similar to the affini-

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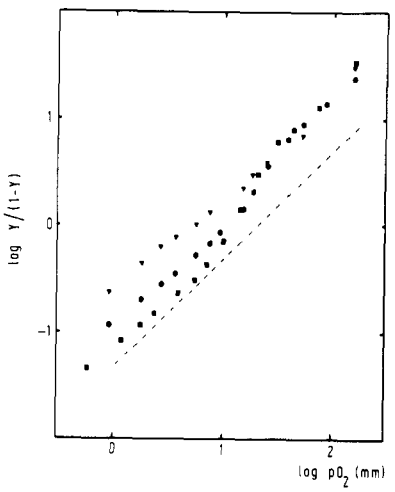

FIGURE 1: Hill plot of oxygen-binding curves in Tris-HCl buffer (pH 8.2), at 20°: (▼) in the presence of 25 mM EDTA, ionic strength 0.1; (●) 25 mM EDTA, ionic strength 1.1; (■) in the presence of 10 mM $CaCl_2$ ionic strength 1.1. Y is the fractional saturation, pO_2 is the oxygen pressure. The line has a slope of 1.0.

ty of the deoxyprotein under physiological conditions. The results support earlier suggestions that only the associated system (half or whole molecules) can occur in a state with low oxygen affinity (Van Driel, 1973).

Materials and Methods

Protein Solutions. Helix pomatia α-hemocyanin was isolated and stored as described previously (Siezen and Van Driel, 1973). Immediately before use the protein was dissolved in demineralized water and dialized against buffer of pH 7 and the appropriate ionic strength and EDTA or $CaCl_2$ concentration. The regeneration was carried out as described earlier (Van Driel, 1973). Determinations of protein concentration and oxygen binding curves were performed according to Konings *et al.* (1969). Buffers contained 50 mM Tris; NaCl was added to obtain the required ionic strength (Bates, 1954).

Analytical Ultracentrifugation. Sedimentation velocity experiments were carried out with a Beckman Spinco Model E analytical ultracentrifuge, equipped with an RTIC temperature control system, photoelectric scanner optics with monochromator, and Schlieren optics. In a typical experiment part of the protein solution was used to determine the oxygen binding curve in duplicate or triplicate, while another part was deoxygenated in a tonometer by flushing with pure nitrogen, and was used for sedimentation velocity experiments at various oxygen concentrations. The oxygen-free protein was incubated for 24 hr at 20° before all measurements. The oxygen concentration in the tonometer was increased stepwise by injecting small amounts of air through a rubber cap on the tonometer. Finally the tonometer was flushed with pure oxygen. After each increase in oxygen concentration the protein solution was equilibrated for at least 15 min, after which the absorbance at 346 nm was read in a Zeiss PMQ II spectrophotometer. Solutions of oxygen-free or partially oxygenated hemocyanin were transferred to one sector of a double sector ultracentrifuge cell as described before (Siezen, 1973). The other sector was filled with oxygen-free buffer. During each sedimentation run a Schlieren photograph and a scan at 346 nm were made simultaneously. This was repeated twice during the run. The weight fraction of each dissociation state of the protein was determined from the area under the Schlieren peaks, and was corrected for radial dilution. Johnston-Ogston corrections were neglected. Material sedimenting between the boundaries of the half and one-tenth molecules was added to the weight fraction of one-tenth molecules. The overall fractional oxygen saturation of hemocyanin during centrifugation was determined from the scans at 346 nm. Since the relation between the fractional saturation and the oxygen pressure is given by the oxygen-binding curve, the oxygen pressure inside the cell could be estimated.

Results

Oxygen-Binding Curves. Figure 1 shows the Hill plots of the oxygen-binding equilibrium curves at pH 8.2, at ionic strength 1.1, in the presence of 10 mM $CaCl_2$, and at ionic strength 0.1 and 1.1, in the presence of 25 mM EDTA, which was added to bind possible traces of divalent cations. At low ionic strength and in the absence of $CaCl_2$, oxygen binding is noncooperative. The Hill coefficient (n_H) is about 0.85. At the same ionic strength, in the presence of 10 mM $CaCl_2$, binding was reported to be highly cooperative, n_H = 4–4.5 (Van Driel, 1973). At ionic strength 1.1 ligand binding is cooperative, both in the absence and presence of $CaCl_2$. The Hill coefficients in the lower, middle, and upper ranges of the curves are about 0.8, 1.8, and 1.0, respectively. The presence of 10 mM $CaCl_2$ has no profound effect on the binding properties at high ionic strength.

Oxygen-Linked Association–Dissociation. In the *absence of calcium ions* at pH 8.2, oxygenated hemocyanin is completely dissociated into one-tenth subunits, both at low and high ionic strength, At ionic strength 0.1 the dissociation state is independent of oxygen concentration. At ionic strength 1.1 65% of the protein associates to half molecules upon deoxygenation, while 35% remains dissociated. Some material is present which sediments slightly faster than the one-tenth subunits; this probably represents a small amount of dimers of one-tenth subunits, which have been observed previously upon association of one-tenth subunits (Siezen, 1973). Starting from deoxygenated protein, that was left to associate for 24 hr, sedimentation analysis was carried out at various oxygen concentrations. In Figure 2a the weight fraction of one-tenth subunits is plotted against the oxygen concentration. Up to 6 mm oxygen pressure (Y = 0.3) the dissociation state does not change. At higher oxygen concentrations the weight fraction one-tenth subunits increases rapidly. The dissociation of half molecules in complete at 30 mm oxygen pressure (Y = 0.8). The fractional oxygen saturation and hence the oxygen concentration were always somewhat lower in the cell than in the tonometer. The observed difference is indicated by a horizontal bar in Figure 2.

At ionic strength 1.1, *in the presence of 10 mM $CaCl_2$,* the oxygenated protein is largely dissociated into one-tenth subunits. About 15% half molecules are present. Under oxygen-free conditions 80% is associated to half and whole molecules and 20% is still dissociated. Figure 2b shows that an abrupt dissociation takes place between 6 and 30 mm oxygen pressure. No attempt was made to differentiate between half and whole molecules, because their relative amounts varied in different experiments. However, the sum of the half and whole molecules is a reproducible parameter. The boundaries between the half and one-tenth molecules are not completely separated. This is probably caused by small amounts of di-, tri-, and tetramers of one-tenth subunits. Sometimes three small peaks in the Schlieren pat-

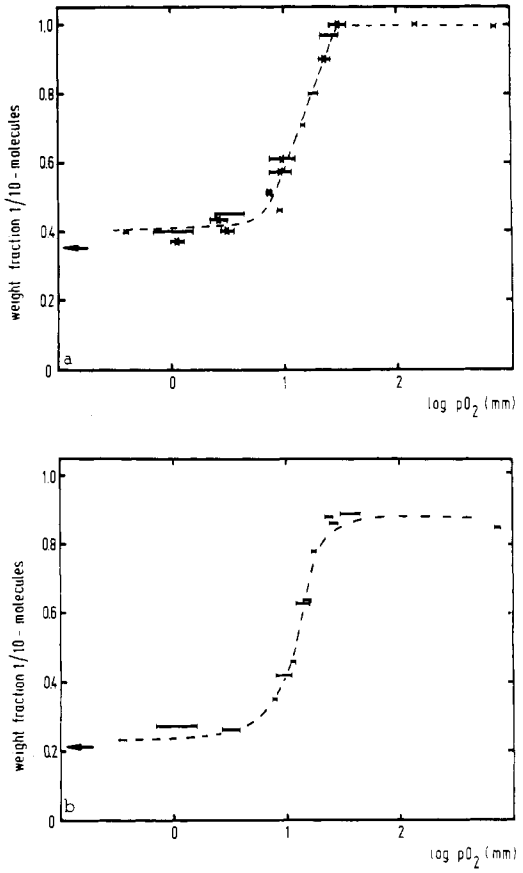

FIGURE 2: Dependence of the weight fraction one-tenth subunits on the oxygen pressure (pO_2); Tris-HCl buffer, ionic strength 1.1, pH 8.2, 20°: (a) 25 mM EDTA, (⊢⊣) and (⊢——⊣) refer to different experiments; (b) 10 mM $CaCl_2$. The arrow indicates the weight fraction at zero oxygen concentration. The horizontal bars represent the drop in oxygen pressure upon transferring the protein solution from the tonometer to the ultracentrifuge cell.

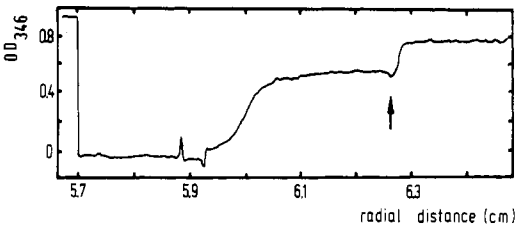

FIGURE 3: Typical scanner trace at 346 nm. Conditions as in Figure 2a, oxygen pressure 7 mm, overall fractional saturation $Y = 0.4$, after 13 min of sedimentation at 37,020 rpm. Note the dip in the scan, indicated by the arrow.

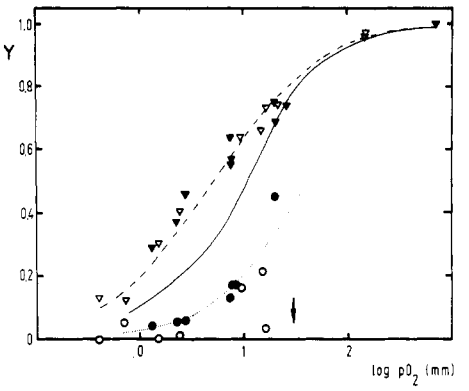

FIGURE 4: Oxygen binding properties of one-tenth and half molecules at pH 8.2, ionic strength 1.1, 25 mM EDTA, at 20°. (▽) and (○) indicate the fractional oxygen saturation of the one-tenth and half molecules, respectively, present at the corresponding oxygen pressure (pO_2). Open and filled symbols refer to different experiments. The curve (—) represents the overall oxygen binding curve at pH 8.2, ionic strength 1.1, 25 mM EDTA (same data as in Figure 1); the dashed curve (- - -) shows the oxygen binding of the one-tenth subunits at pH 8.2, ionic strength 0.1, 25 mM EDTA (same data as in Figure 1), the dotted curve (. . .) is a hypothetical noncooperative binding curve with a P_{50} of 40 mm, and the arrow indicates the oxygen pressure where all half molecules have disappeared.

tern can be observed between the boundaries of one-tenth and half molecules. At low ionic strength ($I = 0.1$) only whole molecules are observed in the presence of $CaCl_2$, under both ligand-free and oxygenated conditions.

Oxygen Binding Properties of the Associated and Dissociated Protein. A typical scanner trace is shown in Figure 3. The fractional saturation of the dissociated protein (one-tenth subunits) and of the half molecules was determined as follows. The optical density (OD_{346}) in the plateau region between both boundaries was corrected for radial dilution and divided by the concentration of the dissociated protein, which was determined from the Schlieren pattern and the total protein concentration. The value obtained is the OD_{346} of the dissociated protein at unit concentration. From this value the fractional oxygen saturation of the one-tenth subunits was calculated. This procedure is not possible for the half molecules, because no OD_{346} of completely oxygenated half molecules can be measured. The oxygen saturation of the half molecules at a certain oxygen concentration was calculated from the equation

$$Y_{cell} = tY_t + (1 - t)Y_h$$

Y_{cell} is the overall fractional oxygen saturation in the cell, Y_t and Y_h are the fractional saturations of the one-tenth and half molecules, respectively, and t is the weight fraction of one-tenth subunits present at that oxygen concentration. Since Y_{cell}, Y_t, and t are determined independently, Y_h can be calculated. Y_{cell}, Y_t, and t are the average of three values obtained during the same run.

The fractional saturation of dissociated and associated protein in the absence of $CaCl_2$, as a function of the oxygen pressure, is reported in Figure 4. Note that these data do not represent normal binding curves, because at different oxygen concentrations different populations of molecules are observed.

A quantitative interpretation of the binding properties of the associated and dissociated proteins in the presence of $CaCl_2$ was less feasible, because no plateaus are observed between the sedimentation boundaries of one-tenth and half molecules.

Discussion

Figure 1 shows that oxygen binding is cooperative at ionic strength 1.1, pH 8.2, both in the presence and absence of $CaCl_2$. Under these conditions the binding of oxygen is linked to a change in dissociation state. Under ligand-free conditions, in the absence of $CaCl_2$, 65% of the protein is present as half molecules and 35% as one-tenth molecules. Upon oxygen binding an abrupt dissociation of the half molecules is observed between 6 and 30 mm oxygen pressure, corresponding to a fractional saturation of 0.3 and 0.8, respectively (Figure 2a). At higher oxygen concentrations only dissociated protein is observed. These results indicate that the oxygen affinity of the half molecules is less than

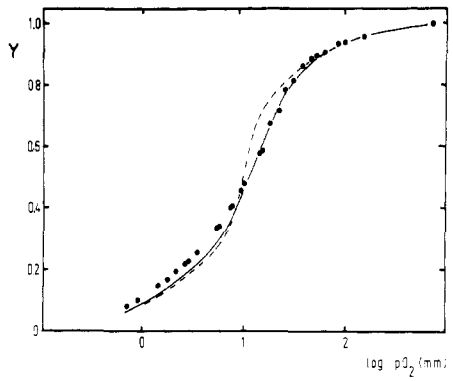

FIGURE 5: Calculated oxygen binding curves. The curve (—) shows the calculated binding curve, assuming that the half molecules bind oxygen noncooperatively (see text); the dashed curve (- - -) represents the calculated binding curve, assuming that the half molecules have the same cooperative oxygen binding properties as reported for whole molecules at pH 8.2, ionic strength 0.1, in the presence of 10 mM $CaCl_2$ (Van Driel, 1973); (●) experimental oxygen binding curve at pH 8.2, ionic strength 1.1, 25 mM EDTA (same data as in Figure 1).

the affinity of the dissociated protein. This difference can be demonstrated more directly by using the photoelectric scanner of the analytical ultracentrifuge. From scans at 346 nm, obtained during sedimentation velocity runs at various oxygen concentrations, the oxygen binding properties of the dissociated protein and the half molecules were estimated, in the absence of $CaCl_2$. The results are shown in Figure 4. The binding properties of one-tenth subunits are similar at low and high ionic strengths; ligand binding is noncooperative; the P_{50} is about 4 mm. The half molecules have about a tenfold lower oxygen affinity; the P_{50} is about 40 mm.

The estimation of the oxygen saturation of the one-tenth and half molecules from the scans is based on the following assumptions: (i) the observed boundaries are true sedimentation boundaries and not reaction boundaries, and (ii) the free oxygen concentration is the same at all places in the ultracentrifuge cell. The first condition is met, because the sedimentation coefficients of the half and one-tenth molecules are the same as those expected for the pure components, and the relative amounts of half and one-tenth molecules do not change during the run. The apparent absence of equilibrium between the associated and dissociated states is due to microheterogeneity. This means that hemocyanin is a population of very similar molecular species, each undergoing a rapid, highly cooperative association-dissociation equilibrium. At any condition, nearly all species will be either completely associated or dissociated; only a minor fraction is in a half-way equilibrium (Siezen and Van Driel, 1973). The observation that the OD_{346} in the plateau regions is constant during the run, after correction for radial dilution, supports the second assumption. At low oxygen concentrations a dip in the scan at 346 nm is observed at the trailing side of the boundary of the half molecules (Figure 3). The dip is absent in scans in the 280-nm absorption band. It indicates that local oxygen gradients may exist.

A detailed analysis of the oxygen binding properties at pH 8.2, ionic strength 1.1, in the presence of 10 mM $CaCl_2$, was not possible. However, the oxygen binding curve and the oxygen-dependent association-dissociation behavior, reported in Figures 1 and 2b, are similar to those observed in the absence of $CaCl_2$. Obviously, in the presence of $CaCl_2$ the dissociated protein also has a high oxygen affinity, whereas the associated protein (whole and half molecules) has a low affinity.

The results suggest that the oxygen binding properties at pH 8.2 and ionic strength 1.1 can be interpreted as an oxygen-linked monomer-pentamer equilibrium between one-tenth subunits and half molecules. This kind of linkage has been analyzed by Wyman (1964). In the case of hemocyanin no rigorous thermodynamic analysis of the system is possible, since the association-dissociation equilibrium is microheterogeneous (Siezen and Van Driel, 1973).

An oxygen binding curve can be calculated, based upon the following assumptions: (i) the ligand binding properties of the one-tenth subunits at low and high ionic strength are identical, (ii) the half molecules bind oxygen noncooperatively with a P_{50} of 40 mm, (iii) Figure 2a represents a state function, relating the fraction of the protein in each dissociation state to the oxygen concentration, and (iv) the overall fractional oxygen saturation is the weight average of the saturation of the components. These simple assumptions give a rather good fit of the experimental binding curve (Figure 5). If it is assumed that the half molecules themselves show intrinsic cooperative oxygen binding, the calculated curve becomes steeper than the experimental curve, as shown in Figure 5. This suggests that the observed cooperativity can result almost completely from the ligand-linked dissociation, in spite of the fact that the associated and dissociated states contain 90 and 18 oxygen binding sites, respectively.

Under more physiological conditions (ionic strength 0.1, pH 8.2, 10 mM $CaCl_2$) oxygen binding is highly cooperative (n_H = 4–4.5). Analysis of the oxygen binding equilibrium showed that cooperativity may be interpreted as an oxygen-linked transition from a state with a low affinity toward oxygen to a state with high oxygen affinity (Er-el *et al.*, 1972; Van Driel, 1973). Under these conditions only whole molecules are observed in both the oxygenated and deoxygenated states. The P_{50} values of the low and high affinity states have been estimated to be 30 and 4 mm, respectively. This is about the same as is found at high ionic strength, at the same pH, in the absence of $CaCl_2$, for the half and one-tenth molecules, respectively. Both the oxygen-linked transition at low ionic strength, and the oxygen-linked dissociation, observed at high ionic strength, take place between about 6 and 30 mm oxygen pressure. It is tempting to conclude that both processes could result from the same molecular mechanism.

It has been found that undissociated, whole molecules can occur in a low oxygen affinity state, characterized by a P_{50} of about 30–40 mm, at pH 8.2 (Van Driel, 1973), whereas dissociated protein (one-tenth molecules) has a high oxygen affinity (P_{50} of about 4 mm) under all conditions investigated. Here we showed that a half molecule also can adopt a conformation with low oxygen affinity. These results suggest that a closed ring of five one-tenth molecules (a half molecule) must be formed before the protein can be constrained to a state with low ligand affinity. Since a low affinity state is a prerequisite for cooperativity, and the half molecule appears to be the smallest unit which can occur in a low affinity state, we suggest that under physiological conditions the half molecule, containing 90 binding sites, can be regarded as the smallest cooperative oxygen binding unit in the whole hemocyanin molecule (containing 180 binding sites).

Oxygen-linked association-dissociation has been reported for other Gastropod hemocyanins as well, particularly for the dimerization of half molecules (DePhillips *et al.*, 1970; Wood and Dalgleish, 1973; Vannoppen-Ver Eecke *et al.*,

1973; Siezen, 1973). In these cases oxygenation favors the associated state. DePhillips *et al.* (1969) showed a complex linkage between ligand binding and the pentamerization of the 19S component of *Loligo* hemocyanin. However, no obvious relation seems to exist between reported cooperativity of oxygen binding and ligand-dependent association–dissociation phenomena.

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Carbon-13 Magnetic Resonance Assignments of the Repeat Peptides of Elastin and Solvent Delineation of Carbonyls

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ABSTRACT: Complete assignments of the carbon-13 resonances, including the peptide carbonyls, are reported for the repeat tetrapeptide, Val_1-Pro_2-Gly_3-Gly_4, the repeat pentapeptide, Val_1-Pro_2-Gly_3-Val_4-Gly_5, and the repeat hexapeptides, Ala_1-Pro_2-Gly_3-Val_4-Gly_5-Val_6, and the cyclic permutation Val_6-Ala_1-Pro_2-Gly_3-Val_4-Gly_5 of elastin. The assignments are given in three different solvent systems, dimethyl-d_6 sulfoxide, trifluoroethanol-d_3, and D_2O. Carbonyl assignments were achieved in each solvent by chemical methods utilizing glycine-*1*-^{13}C enrichment and chemical modification of end residues. Solvent mixture titrations, which further check the carbonyl assignments, are reported and discussed relative to possible conformational information. In a manner similar to peptide NH protons, the peptide C–O carbonyls can be delineated by solvent dependence of chemical shift. In the tetrapeptide and pentapeptide the Val_1 C–O which hydrogen bonds to the Gly_4 NH and Val_4 NH, respectively, in formation of a β turn, is solvent shielded. In the two hexapeptides it is the Ala_1 C–O which hydrogen bonds to the Val_4 NH that is solvent shielded.

Solution studies on the conformation and interactions of elastin have utilized hot oxalic acid fragmentation of the elastin fiber which results in two major products, α-elastin (~70,000) and β-elastin (5–10,000) (Partridge *et al.*, 1955). The α-elastin fragment contains some 16 chains held together by four to five cross-links (Partridge and Davis, 1955).

More recently, the precursor protein, tropoelastin, has been isolated (Smith *et al.*, 1968; Sandberg *et al.*, 1969) and the amino acid sequences of some 13 peptides of tropoelastin which comprise almost one-half of the molecule have been determined (Foster *et al.*, 1973; Gray *et al.*, 1973). Gray and Sandberg and their colleagues report a repeating tetrapeptide (-Gly-Gly-Val-Pro-), a repeating pentapeptide (-Pro-Gly-Val-Gly-Val-), and a repeating hexapeptide (-Pro-Gly-Val-Gly-Val-Ala-). In previous work we have synthesized these peptides and their oligomers and have studied their conformations using proton magnetic resonance methods (Urry, 1974; Urry *et al.*, 1974; Urry and Ohnishi, 1974a,b). All three peptides exhibit a β turn in which the -Pro-Gly- sequence forms the corners, *i.e.*, positions $i + 1$ and $i + 2$, respectively, as indicated in Figure 1.

The present effort reports the carbon-13 assignment of the repeating peptides of elastin with particular emphasis on the carbonyl carbons. The assignments are given in three different solvent systems, *i.e.*, in dimethyl sulfoxide as this solvent pertains to the proton magnetic resonance studies on the repeating peptides, in trifluoroethanol as it is in this sol-

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