Carbon monoxide chlorocruorin from *Eudistylia vancouverii* shows three distinct first-order relaxations with rates of $2.9 \times 10^6$ s$^{-1}$, $6.5 \times 10^5$ s$^{-1}$, and $3.2 \times 10^5$ s$^{-1}$ (geminate reactions) and three second-order relaxations with rates of $4.7 \times 10^6$ M$^{-1}$ s$^{-1}$, $7 \times 10^5$ M$^{-1}$ s$^{-1}$, and $7 \times 10^5$ M$^{-1}$ s$^{-1}$, when studied by flash photolysis. The amplitudes of the second-order reactions depend on the extent of photolysis. This may be due to relaxation from the liganded (R) to the unliganded (T) conformation following photolysis and suggests that the combination rates contribute to cooperativity. In a stopped-flow experiment only the slowest phase with a rate of $7 \times 10^5$ M$^{-1}$ s$^{-1}$ is observed. It is assigned to binding to the T-state protein. Fragments of the native protein containing 12 and 4 hemes react like the holoprotein (10). The amplitudes of the second-order phases do not correlate with the extent of photolysis. The results with the two ligands are consistent with an allosteric transition fast enough to compete with oxygen rebinding (10') in the R to T direction (CO rebinding) but not fast enough to compete with oxygen rebinding. There is significant heterogeneity in the R-state kinetics, but the T-state reaction is homogeneous.

Chlorocruorin is the extracellular oxygen-binding protein found in four polychaete families of the annelid phylum (1). Although its heme has a formyl group instead of the 3-vinyl group of protoheme IX (2), its physical and chemical properties appear to be very similar to those of the ~3,600-kDa, hexagonal bilayer, extracellular hemoglobins of oligochaetes and other polychaetes (3). The chlorocruorin from *Spirographis spallanzanii* has been studied by Antonini, Brunori, and their collaborators (4–6). Analysis of cooperative O$_2$ binding by S. spallanzanii and *Potamilla* chlorocruorins indicates a minimum cooperative unit of 6 or 12 hemes (7, 8). Characterization of the kinetics of O$_2$ and CO binding to S. spallanzanii chlorocruorin has shown signs that quaternary conformation may affect heme reactivity with ligands (9).

A recent, detailed study of the properties and dissociation of the chlorocruorin from *Eudistylia vancouverii* has provided substantial evidence that this molecule, and by implication chlorocruorins in general, consists of 12 ~200-kDa complexes of globin (~16 kDa) chains, linked together by 30–40 chimeric (~30 kDa), heme-deficient globin chains and 30–40 tightly bound Ca(II). The similarity between the proposed quaternary structures of *E. vancouverii* chlorocruorin and of the hemoglobins from *Lumbricus terrestris* and *Macrobdella decora* (11, 12) suggests that the giant, hexagonal bilayer oxygen binding proteins of the annelids may be viewed as hierarchies of globin complexes, where the principal functional and structural subunits are dodecamers of globin chains, themselves made up of smaller globin subunits. In *E. vancouverii* chlorocruorin the structural units are disulfide-bonded tetramers and noncovalent tetrarimers of disulfide-bonded dimers (10).

A recent detailed study of ligand binding kinetics of *L. terrestris* hemoglobin (15) is consistent with the structural proposals. The functional significance of the amino acid residues around the heme pocket of myoglobin has been assessed, to some degree, by site-directed mutagenesis (14). The results allow some interpretation of the rather striking differences between *L. terrestris* and myoglobin and, in particular, suggest that much of the obvious heterogeneity in CO binding by the earthworm hemoglobin may arise from primary structure differences between the subunits. A definite conclusion was that none of the marked cooperativity in equilibrium CO binding can be attributed to allosteric effects on the combination reactions. This paper reports the results of kinetic experiments on the binding of oxygen and carbon monoxide to *E. vancouverii* chlorocruorin and its dodecamer and tetramer subunits in the picosecond to millisecond time range. Although the structural information for the chlorocruorin is less complete than for the earthworm hemoglobin, on the basis of the kinetic results, the functional unit is likely to be of the same size as in the earthworm, but the allosteric origin of cooperativity in the protein and its subunits is considerably different.
described recently (14, 15). Almost all of the data were obtained by flash photolysis which has important advantages in dealing with limited quantities of material and allows repetition of experiments and averaging of the results on the same sample. Averaging is particularly useful with chlorocruorin where ligand binding is not associated with the large changes in the position of the Soret bands typical of hemoglobins but rather with relatively small changes in absorbance. Many experiments were performed using 460 nm as the observing wavelength, except for the picosecond experiments where the limitations of the apparatus allow the use of 436 nm only.

In the microsecond to millisecond range used for following the second-order recombination reactions, 50–200 reactions were averaged, collecting 1024 points to represent each run. The data were reduced to absorbance changes and fitted by non-linear least squares to a single second-order reaction. If the residuals were non-randomly distributed, two reactions were used. In most cases, the residuals, though much smaller, continued to be non-random, but a single trace was insufficiently precise to support the determination of three rates and three amplitudes. Estimates could be obtained by combining data obtained under different conditions of photolysis and ligand concentration for analysis. The reliability of these values is hard to assess, because standard procedures are not valid if any systematic error is present. The data themselves are highly reproducible, and in repeated experiments using different samples the rates and amplitudes agreed within 10–15%. The lowest rate of CO recombination is so slow that the results may be influenced by the photochemical effects of the observation beam. At low concentrations of CO, e.g. 100 μM CO, a photostationary state appreciably removed from equilibrium is set up and the apparent rate and amplitude of the slowest component significantly altered. The experiments were repeated attenuating the observation beam systematically until the photochemical effects disappeared, and, where possible, 100% CO was used in the gas phase.

RESULTS

Second-order Reaction of Carbon Monoxide—Carbon monoxide binding to the fully reduced protein was followed in the stopped-flow apparatus of Gibson and Milnes (16). The reaction accelerates as it proceeds, so that with low (50 μM) CO, the first-order rate (≈4 s⁻¹) describing the last third of the reaction is some 50% larger than that for the first third. The observed initial rate of binding increases in proportion to the concentration of CO up to 460 μM. The acceleration may reasonably be attributed to an increasing population of rapidly reacting R-state as the reaction proceeds, and the initial rate identified with the slowly reacting T-state. The initial rate corresponds to a second-order rate of 6×10⁻⁴ M⁻¹ s⁻¹. These points are illustrated in Fig. 1, panel D.

The stopped-flow experiment starts with T-state unliganded protein, whereas flash experiments start with the liganded species, usually in the R-state. In the flash apparatus, data were first collected using flashes of 9-ns duration from the YAG laser, following the reaction for times up to a tenth of a second. Even with the brightest flashes available to us only two-thirds of the total bound carbon monoxide was freed from the protein to take part in the second-order recombination reaction, as judged by comparing the kinetic and static difference spectra. This is not due to lack of sufficient quanta, as control experiments showed that MbCO was more than half dissociated when the flash intensity was reduced 64-fold. The ratio of the extinction coefficients of the two proteins at 532 nm is 0.8 with MbCO being the greater. This difference is too small to allow the effect to be explained by more efficient trapping of quanta by MbCO. Further, at the higher light levels, there was little change in the absorbance excursion as the flash increased in intensity.

Many possible causes of this result are excluded by the observation that a much larger proportion of CO, approaching 1, became available for the second-order reaction when a flash of 300 ns, obtained from a dye laser, was used for photolysis. This is readily explained by supposing that a significant fraction of the ligand which has been dissociated from the heme recombines in a first-order reaction too fast to be observed with the 1-μs time resolution in this experiment. Such a reaction is usually called geminate recombination and has been extensively investigated, particularly for sperm whale myoglobin, e.g. by Ref. 14. The incomplete photolysis of chlorocruorin complicates the interpretation of the second-order time courses observed following flash photolysis. References may be found through a short review (17).

The time course of CO recombination following flash photolysis is described only approximately by two second-order reactions with a 20-fold difference in rate. Although examination of the residuals shows systematic deviations, there is insufficient information in any single record to define a third component. Examination of a group of experimental traces obtained using different concentrations of free carbon monoxide and varying levels of photolysis allowed three rates and amplitudes to be defined with reasonable assurance. The rate for the slowest component was the same as the value observed in stopped-flow experiments, and in subsequent analysis, this value was introduced as a fixed parameter. Two rates and three amplitudes could now be determined readily, chiefly because of the order of magnitude separation between the rate of each component. The ratio of the two faster rates is now less than 10:1 because a single slower rate is no longer required to approximate two disparate steps, each of which is now represented by its own rate parameter.

The second-order rate constants show little or no change with the extent of photolysis and the length of the laser pulse; the relative amplitudes of the phases, however, are influenced by these factors as well as by the ligand concentration, as illustrated in Fig. 1, panels B and C, and Fig. 2. The values of the second-order rates are 4.7×10⁻⁴ M⁻¹ s⁻¹, 7×10⁻⁴ M⁻¹ s⁻¹, and 7×10⁻⁵ M⁻¹ s⁻¹.

The proportion of the absorbance change attributable to the fastest component is independent of the extent of photolysis, while the proportions of the two slower components change systematically (Fig. 2); this is consistent with a contribution to cooperativity from their combination velocity constants analogous with that of vertebrate hemoglobins, with the R-state binding faster than the T-state. The larger the fractional photolysis, the greater the opportunity for population of the T-state and the larger the proportion of the slowest rate of rebinding. The ratio of the amplitudes also depends on the concentration of ligand, again in analogy with the behavior of mammalian hemoglobin, and is explained by competition between ligand rebinding and allosteric rearrangement of chlorocruorin following photodissociation, with a larger proportion of the faster reactions at high ligand concentrations.

Geminate Rebinding of Carbon Monoxide—The observation, reported above, that the 9-ns laser pulse was not able to photolyze the CO complex of E. vancouverii chlorocruorin completely was used as a basis for us to search for ligand rebinding on a shorter time scale. Significant first-order ligand rebinding takes place on a time scale of hundreds of picoseconds to hundreds of nanoseconds. An example of these data is shown in Fig. 3 on a logarithmic time scale. The record begins with a decrease in saturation which occupies some 20 ps and represents photodissociation by the flash. Thereafter, during the next nanosecond, CO rebinding occurs at a rate of 2.9×10⁻⁹ s⁻¹. This result is very unusual, and so far as we are aware, this is the first time that picosecond recombination of carbon monoxide to a heme protein has been reported. This very high rate is followed by two further first-order reactions with rates of 6.5×10⁻⁷ s⁻¹ and 3.2×10⁻⁶ s⁻¹. These reactions are responsible for the points forming a shoulder on the curve.
Fig. 1. Time course of the reaction of carbon monoxide with *E. vancouverii* chlorocruorin. Panel A: laser pulse, 35 ps; observing wavelength, 436 nm. Panels B and C: laser pulse, 9 ns; observing wavelength, 460 nm. Panel D: stopped-flow experiment, observing wavelength, 460 nm. All experiments were run in 0.1 M phosphate buffer, pH 7.0, at 20 °C; the concentration of CO was 920 μM in photolysis experiments and 460 μM in stopped-flow experiments. The path length was 1 mm in photolysis experiments and 2 cm in the stopped-flow run. The protein concentrations were 80 μM in photolysis and 3.5 μM in the stopped flow (after mixing).

Fig. 2. Dependence on the extent of photolysis of the proportions of the three second-order components of the time course of carbon monoxide rebinding. The conditions were the same as for the photolysis experiment of Fig. 1; two flash lengths were used: 9 ns at 532 nm, and 300 ns at 575 nm. The amplitudes have been scaled to 1 for each photolysis level and were obtained by non-linear least squares fitting using a single set of three rate constants of 4.7 × 10^6 M^-1 s^-1, 7.4 × 10^6 M^-1 s^-1, and 7.2 × 10^4 M^-1 s^-1. The symbols are: ■, fastest rate; □, intermediate rate; ○, slowest rate.

and filling the time interval between the nanosecond and microsecond observations (second order), which are also included in Fig. 3. As already stated, the definition of so many rates is possible only because they are so widely separated.

It is important to note that the total absorbance change calculated from the data of Fig. 3 (which is a composite of experiments using 436 and 455 nm for observation) agrees within 5% with the values expected from static spectra. The geminate amplitudes are also sufficient to account for the effects of flash length in the experiments on a millisecond time scale discussed in the previous paragraph. Comparisons between chlorocruorin and sperm whale myoglobin CO, used as a standard, point to a quantum yield for the initial photoreaction of 1.

Fig. 3. Extended time course of carbon monoxide rebinding. The ordinate is absorbance, and the abscissa is the logarithm of time in picoseconds. A flash of 35 ps started to remove CO at A. The flash was complete, and geminate rebinding began at B. Three geminate phases follow, C, D, and E, together extending to 0.5 μs. Data representing second-order rebinding are shown by F, G, and H.

Second-order Oxygen Rebinding to Chlorocruorin—Interpretation of the time course of oxygen rebinding after photolysis (9-ns flash, 532 nm) is more difficult than for carbon monoxide, because the relatively rapid spontaneous dissociation reaction must be considered. In addition, the effective quantum yield, as with other oxy hemoproteins, is low, and full dissociation cannot be closely approached. Finally, the affinity for oxygen is low, and the chlorocruorin is not fully saturated in experiments with low O₂ tensions. For these reasons, only a phenomenological description of oxygen rebinding can be given. In spite of these limitations the differences from the behavior with carbon monoxide allow some conclusions to be drawn.

Data were collected, by flash photolysis, for three oxygen concentrations, 1.2, 0.25, and 0.05 mM. In each case, the time course of rebinding was well represented by a sum of two exponentials (because of the small extent of photodissociation
Chlorocruorin of *E. vancouverii*

the concentration of free \([O_2]\) does not vary significantly during rebinding). The rates correspond to second-order reactions of approximately \(1.5 \times 10^7 \text{ M}^{-1} \text{s}^{-1}\) and \(1 \times 10^7 \text{ M}^{-1} \text{s}^{-1}\) in the simplest case of two independent reversible reactions, plotting the observed exponential rates against the concentration of free oxygen should give straight lines intercepting the ordinate at the rate of oxygen dissociation. The lines did not intercept the ordinate, however, but the abscissa. This may be due to several causes; one is that at the lower oxygen concentrations the protein is not fully saturated. With 0.05 mM oxygen, the measured saturation was only 11%, and it is likely that the allosteric population would differ from that at higher saturations. If the measured rates reflect binding more than dissociation, an increasing proportion of T-state would contribute a lower rate and a downward trend in the plot of rate versus oxygen concentration.

The distribution of fast and slow phases at different oxygen concentrations and different degrees of photolysis, unlike the result with carbon monoxide, did not show a consistent trend. This would happen if the rate of the allosteric transition were lower than the rate of ligand rebinding and is consistent with substantial population of a liganded T-state at the lowest oxygen concentrations.

**Geminate Reactions with Oxygen**—Rapid rebinding of oxygen to *E. vancouverii* chlorocruorin may be described by three first-order rates of \(2.5 \times 10^9 \text{ s}^{-1}\), \(3 \times 10^8 \text{ s}^{-1}\), and \(4.5 \times 10^7 \text{ s}^{-1}\). The first phase was followed at 436 nm using the pulse probe apparatus and accounts for over 40% of the total absorbance change (Fig. 4, panel A). It appears that some other rapid process competes with the flash, since the apparent quantum yield at the peak of the pulse (0.4) is significantly less than 1.

The two slower phases are more readily followed after the longer laser flash (9 ns); their rates are similar to those describing the rebinding of carbon monoxide. Their amplitudes (Fig. 4, panels B and C), however, are considerably different, the faster phase in oxygen rebinding accounting for a larger fraction of the total absorbance change than in the corresponding CO reaction. The two oxygen nanosecond reactions, like those of CO, show some dependence on the extent of photolysis. As expected from the amplitude of the geminate reactions, the apparent quantum yield for oxygen is much smaller than that for CO.

**Difference Spectra**—The static spectra of the reduced, oxy, and carbonmonoxy derivatives are closely similar to those reported for *S. spallanzanii* (4). Kinetic difference spectra in the nanosecond and microsecond time ranges agreed, within experimental error, with one another and with the static spectra.

**Reactions of Dodecamer and Tetramer with Carbon Monoxide**—The dodecamer corresponds to one-eighth of the native molecule and the tetramer to one-twenty-fourth; neither species occurs naturally. The kinetics of carbon monoxide binding to these smaller assemblies has been compared with that of native molecule. The tetramer and dodecamer are similar to one another, and to the native chlorocruorin, all showing geminate reactions with carbon monoxide in the picosecond and nanosecond time regimes following flash photolysis, as well as triphasic second-order rebinding. The second-order rates are the same for both tetramer and dodecamer (\(4.5 \times 10^7 \text{ M}^{-1} \text{s}^{-1}\), \(9 \times 10^6 \text{ M}^{-1} \text{s}^{-1}\), and \(5.5 \times 10^5 \text{ M}^{-1} \text{s}^{-1}\)) and agree closely with those of the native chlorocruorin. Moreover, their relative proportions depend on the extent of photolysis, suggesting that cooperative ligand binding occurs in the smaller assemblies.

A stopped-flow experiment, carried out under the same experimental conditions, gave a single second-order process, with a rate of \(6 \times 10^4 \text{ M}^{-1} \text{s}^{-1}\) for both species, showing that, at equilibrium, the deoxy dodecamer and tetramer have kinetic properties similar to those of the slowest component observed in flash photolysis. The difference in rates of CO binding measured in the stopped-flow and flash experiments shows that these smaller units make a functional (R-T) transition analogous with that of the holoprotein. Briefly, the dodecamer and tetramer can scarcely be distinguished from the native protein.

**DISCUSSION**

Chlorocruorin from *E. vancouverii*, exceptionally, shows extensive geminate rebinding of CO on both picosecond and
nanosecond time scales. Although rapid binding of CO to free heme has been reported (20) with a first-order rate of the order of \(10^9\) s\(^{-1}\), the small geminate reaction of most heme-proteins is of the order of \(10^7\) s\(^{-1}\); thus the observed rate for chlorocruorin, in excess of \(3 \times 10^8\) s\(^{-1}\), is significantly faster. Although the difference spectrum for the picosecond process cannot be obtained with our apparatus, the effective quantum yield is near unity, and the amplitude of the absorbed absorbance change is consistent with the static difference spectrum. This high rate is more likely to be due to the protein structure confining the ligand close to the heme than to the chemical difference between protoporphyrin IX and heme \(a\) since Blackmore et al. (21), who recently reported geminate recombination of NO in cytochrome oxidase, did not observe an analogous reaction with CO.

The carbon monoxide derivatives of myoglobins show relatively little recombination of ligand on a sub-microsecond time scale, a result consistent with, and indeed required by, the observation that almost every photon absorbed by the protein brings about escape of a carbon monoxide molecule to the solvent, i.e. the quantum yield for the whole process is near unity (18, 19).

The oxygen reaction of myoglobins is quite different; there is usually extensive (geminate) recombination of ligand in the sub-microsecond range, and the effective quantum yield for escape of ligand to solution is correspondingly low, e.g. 0.1 for sperm whale myoglobin (19).

The second-order reaction of chlorocruorin with carbon monoxide is complex and can be simulated only by assuming that the protein offers at least two heme environments in the R-state with different rates of ligand binding and that at least one (and probably both) of them convert to a (common) T-state following ligand removal.

This is consistent with the data of Fig. 2 which shows that the proportion of the slowest (T-state) reaction increases with increasing photolysis, while at the same time the proportion of the intermediate (R-state) reaction decreases. The implication is that there is an allosteric transition able to compete with CO re-binding to this component. In contrast, the proportion of the fastest component does not change in these experiments, but slow conversion to the T-state must take place in longer times, as the stopped-flow experiments show no rapid component. The reaction of chlorocruorin with oxygen is somewhat more complex. The apparent quantum yield of 0.4 at the peak of the 35-ps laser flash suggests a very fast reaction similar to that observed in the oxygen and nitric oxide reactions of myoglobins (22), though to a lesser extent. The first observed picosecond reaction at \(2 \times 10^{10}\) s\(^{-1}\) accounts for about a third of the total absorbance change observed in the pulse probe experiment. At the highest light levels, cumulative bleaching of the protein is seen, indicating irreversible photochemical damage to the protein, as has been observed by several investigators (e.g. Ref. 22) for myoglobin. The \(2 \times 10^{10}\) s\(^{-1}\) component has too long a lifetime to be attributed to the ultrafast intermediate of Petrich et al. (22) which has a half-life of only 3 ps and is, therefore, by default, attributed to recombination of oxygen, although our probe-pulse apparatus did not allow this to be confirmed by measuring difference spectra. The nanosecond geminate reactions of oxygen account for a larger fraction of the total recombination reaction than the corresponding reactions of carbon monoxide but have similar rates.

The proportions of the second-order reactions with oxygen do not change with photolysis intensity, presumably because the allosteric change is not fast enough to compete effectively with re-binding. It is also possible that the rates of oxygen binding to the allosteric states differ less than those for carbon monoxide, as is the case for mammalian hemoglobins. It is reasonable that the rates for carbon monoxide binding to the tetramer and dodecamer are so similar to those of the whole assembly, as the slow binding of this ligand is unlikely to be limited by diffusion through the protein. These experiments show, primarily, that the structures around the heme are preserved in the smaller units. It does appear, however, that they are cooperative. This matter would be better studied by equilibrium methods.

The chlorocruorin from \(E.\) vancoeurui differs from the giant molecule from the earthworm (15), believed to have a similar structure, in that binding kinetics contribute to its cooperativity. It should be stressed, however, that our experiments do not permit any conclusions to be drawn about possible R-T differences in the rates of ligand dissociation. There are also important similarities. Chlorocruorin shows at least three rates in carbon monoxide rebinding, as the earthworm pigment does also. In the earthworm pigment the heterogeneity can plausibly be associated with unusual amino acid substitutions near the heme group. The necessary sequence information is not yet available for the chlorocruorin.

Cooperativity is retained by the dodecamer subunits of both pigments and also by the tetramer from chlorocruorin. Evidently, much remains to be done with this protein; the relative complexity of its kinetic behavior poses questions to be answered by future work on its composition and structure.

REFERENCES