Activation of Heavy Meromyosin Adenosine Triphosphatase by Various States of Actin†

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ABSTRACT: F-actin monomer (F-monomer) is formed upon the addition of neutral salt to G-actin. Since F-monomer has a digestibility similar to that of F-actin and much lower than that of G-actin, it has been proposed that F-monomer has a conformation different from that of G-actin and similar to the conformation of the subunits in F-actin. To examine whether F-monomer will enhance the magnesium-activated myosin adenosine triphosphatase (Mg^{2+}-ATPase) as much as F-actin, the ability of partially polymerized actin populations at equilibrium to activate the Mg^{2+}-ATPase of heavy meromyosin was investigated. Correlations were made between ATPase activities and the polymerization state of actin as determined by measurements of viscosity and digestibility. No significant activation of the heavy meromyosin ATPase was observed under conditions where G-actin or mixtures of G-actin and F-monomer were present. As polymer formation occurred at higher actin concentrations, or with increased KCl concentrations, substantial activation characteristic of F-actin was observed. The data suggest that F-monomer may undergo a further conformational change as it forms nuclei or joins onto polymers. Alternatively, the site of actin which activates the myosin ATPase may involve the crevice between two adjacent actin subunits.

It is common knowledge that F-actin will bind and greatly activate the Mg^{2+}-ATPase of myosin or its proteolytic digestion products HMM and S-1.[1] However, the polymeric nature of F-actin has limited our understanding of the structural or conformational state of actin that is necessary for participation in the actin–myosin interaction. Monomeric G-actin, which can be more readily studied, also interacts with HMM and S-1, and, while only a 1:1 complex appears to be formed between G-actin and S-1, heavy meromyosin has been reported to promote the polymerization of G-actin (Martonosi & Gouvea, 1961; Yagi et al., 1965; Tawada & Oosawa, 1969a,b; Kikuchi et al., 1969; Cooke & Morales, 1971). In contrast to F-actin, G-actin shows little ability to activate the Mg^{2+}-ATPase of S-1; at very low ionic strength, a 1.6-fold activation was measured at 25 °C (Offer et al., 1972), while matrix-bound G-actin, which is unable to polymerize, activates the S-1 ATPase about twofold (Chantler & Gratzer, 1973, 1976). Since it has been established that G-actin undergoes a change in conformation upon polymerization (Higashi & Oosawa, 1965; West, 1970; Stone et al., 1970; Murphy, 1971; Lehrer & Kerwar, 1972), it is possible that the change in tertiary structure of G-actin upon being transformed into a subunit of F-actin accounts for the large increase in ATPase-activating ability.

Actin polymerization is considered to be a cooperative condensation reaction that proceeds in two steps: (1) the slow formation of F-actin nuclei, which has a third or fourth order dependence on actin concentration implying that three or four monomers must join to form a nucleus; and (2) the rapid growth or extension of nuclei into polymers, which has a first-order dependence on actin concentration (Oosawa et al., 1959; Oosawa & Kasai, 1962; Kasai et al., 1962b; Asakura et al., 1963). Recently a study of the susceptibility of actin to enzymatic proteolysis indicated that another polymerization intermediate is formed after addition of neutral salt to G-actin and *prior* to nucleation or polymer formation (Rich & Estes, 1976). This newly detected state was judged to be monomeric from its low viscosity, its possession of ATP as a bound nucleotide, and its inability to enhance polymerization when added to a slowly polymerizing actin population. It was called F-actin monomer or F-monomer, since its susceptibility to proteolytic digestion is much lower than that of G-actin and similar to the low digestion rate of F-actin. The proposal was made that the conformation of F-monomer is similar to the conformation of the subunit in F-actin.

To further examine this possibility, the current comparison of the abilities of the various known states of actin to activate the Mg^{2+}-ATPase of HMM was undertaken. The results show that neither F-monomer nor G-actin significantly activates the HMM ATPase. This suggests that the conformation of F-monomer may undergo a further change as it joins onto nuclei or growing polymers, or alternatively, that the site of actin which activates the myosin ATPase may be located in the crevice or junction between two adjacent actin subunits.

Methods

Actin was extracted from rabbit acetone powder (Szent-Gyorgyi, 1951) and purified by previously published procedures (Rich & Estes, 1976), except that, after overnight polymerization of the crude extract in 0.05 M KCl and 0.002 M $MgCl_2$, the actin was made 0.8 M in KCl and incubated at 0 °C for 90 min before centrifugation at 170 000*g* for 2 h (Spudich & Watt, 1971). Myosin was prepared according to the procedure of Kielley & Harrington (1960) and HMM from the myosin by the method of Eisenberg & Moos (1967) except that the myosin was digested for 5 instead of 4 min. In order to reduce the amount of KCl added along with HMM to the experimental samples, the purified HMM was further dialyzed against two changes of 0.01 M KCl. Protein concentration determinations were accomplished as previously described for actin (Rich & Estes, 1976) and for HMM (Eisenberg & Moos,

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[1] Abbreviations used: Mg^{2+}-ATPase, magnesium-activated adenosine triphosphatase; HMM, heavy meromyosin; S-1, heavy meromyosin subfragment 1.

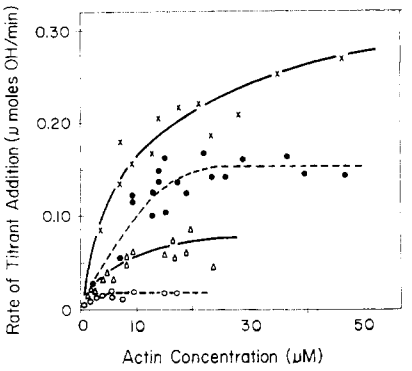

FIGURE 1: Rates of enzymatic proteolysis of equilibrium actin solutions at various concentrations of KCl. Portions of the same samples used in Figures 2 and 3 were digested by subtilisin (final concentration, 0.04 mg/mL) at 25 °C. The rates of titrant addition were determined from the initial linear rate of addition of 10 mM NaOH. (X—X) No KCl (G-actin); (●- - -●) 5 mM KCl; (△—△) 10 mM KCl; (○- - -○) 20 mM KCl.

1967). For calculation purposes, molecular weights of 43 000 for monomeric actin and 350 000 for HMM were used.

In all cases, the actin samples were prepared by diluting G-actin to the desired concentration in such a way that the appropriate final concentration of KCl was present as well as final concentrations of 0.5 mM ATP, 0.01 mM $MgCl_2$, and 2 mM Tris, pH 7.8. All solutions used in sample preparation were filtered through 0.45-μm filters into sterile vessels to prevent bacterial growth during incubation. Each actin sample was then allowed to reach equilibrium for at least 18 h at 25 °C before portions were removed for measurements of viscosity and of rate of digestion by subtilisin (Rich & Estes, 1976). To other portions of the sample, HMM was added to a final concentration of 0.36 μM and the ATPase activity measured at 25 °C (Eisenberg & Moos, 1967). After these initial measurements were completed, the $MgCl_2$ concentration in a portion of each sample was increased to 3 mM to induce polymerization. The viscosity and ATPase-activation measurements on these fully polymerized portions are shown only for the 5 mM KCl conditions (Figure 3b), where denaturation is most likely to occur, but all incubated actin samples were found to be fully polymerized upon addition of $MgCl_2$, demonstrating that the incubation conditions did not cause significant denaturation.

Results

F-monomer only exists as a stable population in the presence of salt below the critical actin concentration, which in 100 mM KCl is in the range 0.5–1.5 μM (Kasai et al., 1962a; Cooke, 1975; Rich & Estes, 1976). Measurement of the activation of HMM ATPase by such low actin concentrations is experimentally difficult, and thus the investigation was conducted at low KCl concentrations where both the critical actin concentration and the amount of actin activation per nmol of HMM are higher (Kasai et al., 1962a; Eisenberg & Moos, 1967).

To aid in identifying the various states of actin present in the partially polymerized actin populations at equilibrium, the susceptibility to enzymatic proteolysis was determined for each actin sample whose viscosity and ATPase-activating ability are shown in Figures 2 and 3. The rates of digestion of various concentrations of actin in 20, 10, and 5 mM KCl can be seen in Figure 1 along with the rate of digestion of G-actin (no KCl present). Subtilisin was the proteolytic enzyme employed because its activity is readily measurable under these conditions (Rich & Estes, 1976). The digestion rates were highest when G-actin was the substrate. As the KCl concentration in the equilibrium actin samples was increased, the magnitude of the digestion rates decreased, and the plateau in the measured digestion rates was attained at a lower substrate (actin) concentration. Previously it was shown that increasing the KCl concentration does not alter the activity of subtilisin enough to account for these changes in the digestion rates (Rich & Estes, 1976). At the lower actin concentrations in 5 mM KCl, the rates of digestion were about half those obtained in the absence of KCl, indicating that the actin population was about one half G-actin. At higher actin concentrations in 5 mM KCl, the digestion rates appear to plateau and become essentially independent of the actin concentration, suggesting that these equilibrium samples contained populations of nearly indigestible F-actin monomers and polymers in addition to readily digestible G-actin (Rich & Estes, 1976). By extrapolation of this plateau back to the G-actin digestion curve, the concentration of G-actin can be estimated to be 10 μM under conditions where the total actin concentration exceeds 25 μM. The rates of digestion of actin samples polymerized in the presence of 10 mM KCl also increase in an approximately linear manner with increasing concentrations of actin but only up to about 10 μM actin, after which they were found to be essentially constant. The lower values for the rates of digestion of actin in 10 mM KCl are evidence that a smaller concentration of G-actin, probably about 2 μM, is in equilibrium with F-actin monomer and polymer under these conditions. In the presence of 20 mM KCl, the low rates of digestion were similar to those obtained with actin which was fully polymerized in 100 mM KCl (Rich & Estes, 1976), and thus these actin populations must be mixtures of mostly F-monomer and F-actin.

The specific viscosity values for equilibrium solutions of various concentrations of actin polymerized in 20 mM KCl and 0.01 mM $MgCl_2$ are shown in Figure 2a along with the ATPase activity of HMM added to a portion of these same solutions. The low specific viscosities at the lower actin concentrations are indicative of a monomer population, but, as the actin concentration was increased above about 1.5 μM (the critical actin concentration), the viscosity values increased in proportion to the amount of polymer present. The ATPase activities of HMM added to the lowest actin concentrations are activated only about twofold, while much higher activities were obtained at the higher actin concentrations. The actin concentration at which the ATPase activities begin to increase is difficult to establish with the low ATPase rates obtained, but it appears to be the same or perhaps slightly lower than that obtained with viscosity measurements. Since the rate of digestion of these same actin samples in 20 mM KCl is low (Figure 1) and similar to that observed for fully polymerized F-actin, the actin population must contain very little G-actin and thus consist predominantly of polymer in equilibrium with F-monomer. This conclusion is strengthened by comparing the ATPase activities of HMM added to the actin samples incubated in 20 mM KCl for 18 h (dashed line, Figure 2a) with the ATPase activity of HMM added to fully polymerized F-actin immediately after being diluted to the same concentrations and conditions and assayed before detectable amounts of depolymerization could occur (dotted line, Figure 2a). Both samples of actin show parallel increases in ATPase activity with increasing actin concentration, and the two curves, on the average, differ only about 0.15 μM actin. This value probably represents the concentration of monomer in equilibrium with F-actin in the incubated actin samples since the amount of monomer in the diluted F-actin samples can be shown by calculation to be quite small. Thus, at the lowest actin concentrations examined, a negligible amount of activation appears to occur with F-monomer.

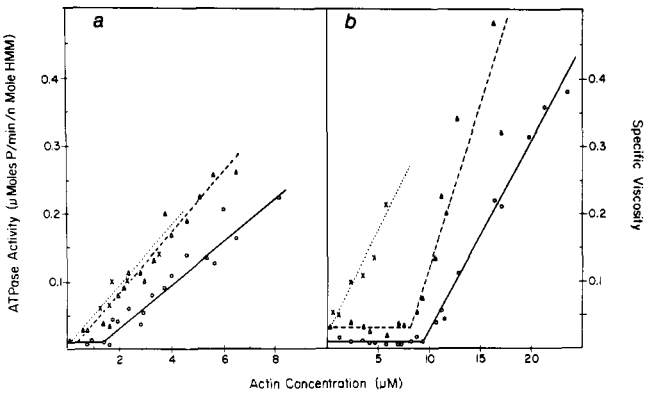

FIGURE 2: Activation of HMM ATPase and specific viscosity of various concentrations of actin polymerized in 20 mM KCl (a) or 10 mM KCl (b). Samples were prepared and polymerized as described in Methods. (O) Specific viscosity; (Δ) ATPase activity; (X · · · X) ATPase activity with fully polymerized F-actin diluted to the indicated concentrations and conditions immediately prior to the addition of HMM to a final concentration of 0.36 μM.

This lack of significant activation of the HMM ATPase by monomeric actin is more readily seen in the same type of experiment with 10 mM KCl conditions shown in Figure 2b. Here the critical actin concentration as determined by viscosity is approximately 10 μM, while the ATPase activity of HMM added to these samples is activated only twofold or less up to an actin concentration of about 8 μM before rising sharply as the actin concentration is raised. The ATPase activities of HMM added to both fully polymerized F-actin diluted to these conditions and to actin polymerized by incubation in 10 mM KCl showed essentially parallel increases as the actin concentration was raised, demonstrating that the incubated actin samples above a concentration of about 8 μM contain a polymer population. Since the viscosity values for actin below concentrations of about 10 μM indicate a monomer population is present, and since the rates of digestion of portions of these same samples have values intermediate between the high rate of digestion of G-actin and the low rate of digestion of F-monomer (Figure 1), the actin present in the region where there is little or no ATPase activation appears to be a mixture of both G-actin and F-monomer. The amounts of each of these states must increase as the actin concentration is raised to around 10 μM, where a transition occurs and the digestibilities become essentially independent of the total concentration of actin present. Above 10 μM actin, it appears that the concentrations of G-actin and F-monomer in each sample remain constant with any increase in total actin forming only F-actin. The less than twofold activation by the monomeric actin population below 8 μM clearly demonstrates that F-monomer does not significantly activate the HMM Mg^{2+}-ATPase activity. This lack of activation by the incubated actin samples below the critical actin concentration in both 20 mM and 10 mM KCl is not due to sample denaturation (see Methods).

In Figure 3a the same type of experiment is again shown but with 5 mM KCl present. Here the viscosity values gradually increase in a manner indicative of a monomer population up to an actin concentration around 28 μM before abruptly increasing due to polymer formation. The ATPase activities remain essentially unactivated until about 25 μM actin after which significant activation is observed. The ATPase activities of HMM added to diluted F-actin again paralleled the activation attributable to the F-actin formed by incubation in 5 mM KCl. These viscosity data and the results of the proteolytic digestion studies (Figure 1) indicate that, in 5 mM KCl for total actin concentrations less than 25 μM, the samples must

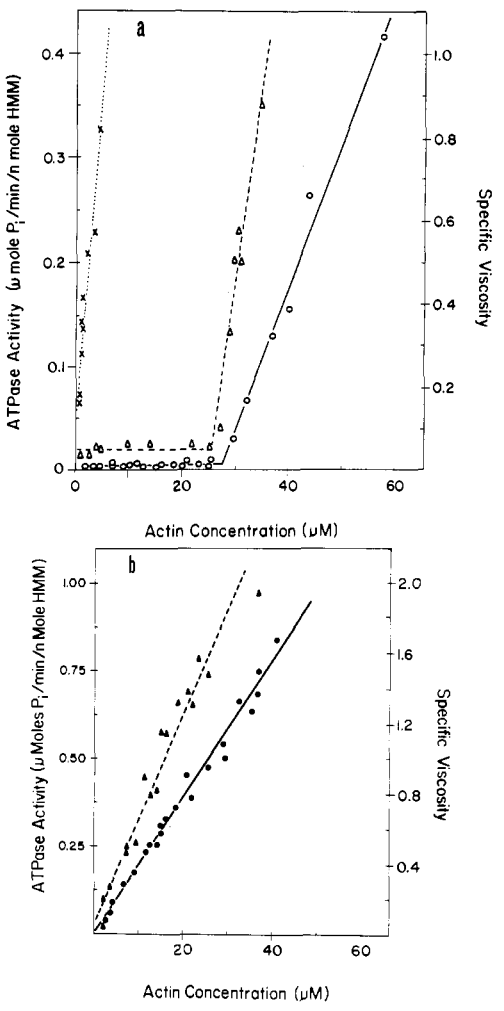

FIGURE 3: Activation of HMM ATPase and specific viscosity of various concentrations of actin polymerized in 5 mM KCl. Samples were prepared and polymerized as described in Methods. (O and ●) Specific viscosity; (Δ and ▲) ATPase activity. (a) $MgCl_2$ (0.01 mM) present (open symbols); (X · · · X) ATPase activity with fully polymerized F-actin diluted to the indicated concentrations and conditions immediately prior to the addition of HMM to a final concentration of 0.36 μM; (b) 3 mM $MgCl_2$ present (filled symbols).

contain equilibrium mixtures of both G-actin and F-monomers. The absence of any significant activation by these monomeric states was not due to denaturation of the incubated actin under these conditions because the addition of $MgCl_2$ (3 mM final concentration) to portions of these same samples caused full polymerization as demonstrated by the high ATPase activities and viscosities shown in Figure 3b (see Methods).

Since the value of the critical actin concentration increases as the KCl concentration is lowered, it was decided to investigate whether G-actin at very high concentrations could activate the HMM Mg^{2+}-ATPase. In the presence of less than 1 mM KCl, which was added with HMM, mixtures of G-actin, at concentrations from 0 to 200 μM, and HMM, at a concentration of 0.36 μM, all had ATPase rates which were near 0.022 ± 0.010 μmol of P_i per min per nmol of HMM. Because no significant activation was observed, it was concluded that the conformation of G-actin, like that of F-monomer, does not activate the HMM ATPase.

Discussion

This study demonstrates that actin activation of the HMM ATPase parallels the occurrence of polymeric actin, as indi-

cated by viscosity, and that at concentrations below the critical actin concentration, populations of both G-actin and F-monomer are present which are incapable of activating the HMM ATPase. The finding that monomeric forms of actin do not significantly activate the myosin ATPase is not in strict agreement with the report by Offer et al. (1972) that G-actin activated the S-1 ATPase 1.6-fold at 25 °C. However, it should be noted that under their experimental conditions actin was treated with amounts of salt which we have found adequate to induce nucleation and the formation of small polymers capable of activating the S-1 ATPase. Our results are also at variance with the reported twofold activation of the S-1 ATPase by matrix-bound monomeric actin (Chantler & Gratzer, 1973, 1976). Since the preparation and methods used by these workers are so dissimilar from those employed in the present study, we are unable to resolve the small discrepancy.

The currently proposed mechanism of polymerization of G-actin into F-actin involves two intermediate states: F-actin monomer, formed by G-actin upon addition of neutral salt; and F-actin nuclei, which result from the joining of three or four F-monomers into an aggregate having a structure such that the formation of double-standard helical polymers can quickly occur (Kasai et al., 1962b; Oosawa & Kasai, 1971; Rich & Estes, 1976). The large difference in susceptibility to proteolytic digestion between the two monomeric states is evidence that salt induces a conformational change in G-actin when forming F-monomer. Since the digestibility of F-monomer is similar to that observed with F-actin, it seemed likely that F-monomer would have a conformation similar to that of the subunits in polymeric actin (Rich & Estes, 1976). The present demonstration that F-monomer does not significantly activate the Mg^{2+}-ATPase of HMM suggests that the conformation of F-monomer may not be the same as that of the subunits in F-actin. Such a conclusion implies that another conformational change occurs in F-monomer as it joins with other F-monomers to form nuclei or is added onto the end of a growing polymer. The occurrence of a conformational change at this step in the polymerization reaction has been previously suggested from both ultraviolet spectral studies (Higashi & Oosawa, 1965) and electron paramagnetic resonance studies (Stone et al., 1970) of the polymerization process. In addition, experiments examining the effect of applied hydrostatic pressure on actin polymerization indicate that the polymerization step itself is accompanied by a large volume change of activation, which possibly results from a change in F-monomer conformation (Estes, 1974, 1975).

The lack of significant activation of the HMM ATPase by the monomeric actin states may not be solely attributable to the absence of this polymerization-associated conformational change. It may be that the site of actin which binds one of the HMM "heads", and activates its ATPase, is formed by the crevice or junction between two adjacent subunits, or is only stable when subunits are joined together. This possibility would preclude significant ATPase activation by any monomeric actin conformational state. Such a requirement of neighboring actin subunits for activation was previously suggested to explain the activation of the HMM ATPase activity by copolymers of actin and carboxymethylated actin (Tawada & Oosawa, 1969a).

At the various KCl concentrations employed in this study, the critical actin concentration determined by viscosity measurements was always 1–3 μM higher than the critical actin concentration determined by ATPase activities. This difference is small enough to be attributable to experimental variation, but it was consistently observed. It is possible that in the region of the critical actin concentration, a state of actin exists which appears to be monomeric in viscosity with the viscometers employed, but which has the digestibility and ATPase-activating ability of F-actin. Such a state of actin could be an "oligomeric" state composed of very short polymers which are hydrodynamically similar to monomeric actin and yet possess properties, in particular the ability to activate myosin ATPase, which are more like those envisioned for F-actin nuclei. While the presence of an "oligomeric actin" state may be suggested by the current study, these data are not adequate to confirm its existence.

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Structure of Methemerythrin at 2.8-Å Resolution: Computer Graphics Fit of an Averaged Electron Density Map†

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ABSTRACT: The crystal structure of methemerythrin from *Themiste dyscritum* has been determined at 2.8-Å resolution by single isomorphous replacement techniques combined with anomalous scattering from a K_2HgI_4 derivative. Noncrystallographic symmetry relating the four subunits in the asymmetric unit was used to obtain an average electron density map of the hemerythrin monomer, and a computer graphics system was used to fit a polypeptide model to the electron density. The average map was of sufficient quality to locate most of the amino acid side chains and to confirm the assignment of His-25, His-54, Glu-58, His-73, His-77, His-101, Asp-106, and Tyr-109 as the iron ligands. One of the mercury sites in the heavy atom derivative is located between two Cys-9 residues related by a noncrystallographic twofold axis, although no intersubunit disulfide bond is present in the native structure. The residues responsible for the binding of the subunits to form the octamer are identified.

Low resolution molecular structures for the oxygen-carrying proteins, myohemerythrin (monomer) and hemerythrin (octamer), have been determined for molecules from different species of marine worms (Hendrickson & Ward, 1975; Ward et al., 1975; Stenkamp et al., 1976b). These models show the secondary and tertiary structure of the subunits and the arrangement of the subunits in the octamer, but a detailed view of the proteins sufficient to explain subunit interactions and the mode of binding of small molecules can only be derived from higher resolution electron density maps.

The preliminary results of a 2.8-Å resolution study of hemerythrin from *Themiste dyscritum* have been reported (Stenkamp et al., 1976a) indicating the geometric arrangement of the iron ligands, but polypeptide models were not fit to the electron density because of the effort required and the uncertainties of the locations of amino acid side chains in the map based on a single heavy atom derivative. We have now used the noncrystallographic symmetry relating the four subunits in the asymmetric volume to obtain an improved average electron density map of the hemerythrin monomer and fit a model to this average density with a molecular display system. This paper reports the results of the model fitting and the structure of hemerythrin.

Electron Density Map. Diffraction data for this study were collected on a computer controlled, four-circle diffractometer by the same techniques used for the 5.0-Å resolution investigation (Stenkamp et al., 1976b).

Table I summarizes the crystal and data collection information. Reflections from four crystals, two native and two derivative, were processed to provide a selected set of 9461 unique Friedel pairs out of the ~14 000 to 2.8-Å resolution. The intensities of from 10 to 13 reflections measured at regular time intervals were used to correct the data sets for radiation decay. The data were corrected for absorption by the usual empirical method (Furnas, 1957; North et al., 1968).

After calculating the heavy atom coefficients as suggested by Matthews (1966a), we sought to refine the positions and B values of individual Hg and I atoms so a more definitive identification of the heavy atom complexes binding to the protein could be made. The least-squares refinement of the parameters for the individual atoms was not well behaved, however, presumably because the Hg and I atoms, separated by ~2.7 Å, were not well resolved by the 2.8-Å data set. Residual density in a difference Fourier map calculated with phases from only the Hg atoms suggested that four of the sites correspond to HgI_2 molecules while the other two were either HgI_3^- or HgI_4^{2-} ions. Refinement of the heavy atoms by ΔF maps did not converge rapidly, so we placed single anisotropic Hg atoms at each site and refined by least squares.

We used the refined heavy atom positions to determine phases for the 2.8-Å resolution data set, making use of the Bijvoet differences to resolve the phase ambiguity (Singh & Ramaseshan, 1966; North, 1965; Matthews, 1966b). The overall figure of merit is 0.807. An electron density map was calculated on a grid of 0.87 × 0.87 × 0.81 Å. The quality of the map was sufficient to identify the iron ligands, and to determine the general location of the C^α atoms in each subunit. The four independent subunits in the asymmetric unit allowed the course of the polypeptide backbone to be traced across occasional gaps in the electron density.

The four subunits in the crystallographic asymmetric unit are distributed between the two octamers in the unit cell. Both octamers are located on unique fourfold axes with the crystallographic asymmetric unit composed of four subunits, two from each octamer. The molecule centered on the fourfold axis

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