Fractionation procedure and specific activities of cytochrome c

The values given in the table are averages of five separate experiments.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Total activity</th>
<th>Purification</th>
<th>Yield /%</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Microsomes</td>
<td>1.1</td>
<td>4.2</td>
<td>4,600</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>II. Deoxycholate supernatant, 0-30% (NH₄)₂SO₄ precipitate</td>
<td>0.3</td>
<td>47.5</td>
<td>14,300</td>
<td>11.2</td>
<td>100</td>
</tr>
<tr>
<td>III. High speed supernatant from Fraction II.</td>
<td>0.1</td>
<td>56.0</td>
<td>5,600</td>
<td>15.5</td>
<td>38</td>
</tr>
<tr>
<td>IV. Ether-extracted enzyme, 10-20% (NH₄)₂SO₄ precipitate</td>
<td>0.06</td>
<td>90.0</td>
<td>5,400</td>
<td>21</td>
<td>37</td>
</tr>
</tbody>
</table>

* Measured in milligramoles of substrate converted per mg of protein per hour.

The latter reaction can be studied as an isolated process with the aid of 2,3-iminosqualene, a potent inhibitor of lanosterol formation from 2,3-oxidosqualene (5). Such studies are now in progress.

REFERENCES


Location of the Heme in Horse Heart Ferricytochrome c by X-Ray Diffraction

(Received for publication, April 3, 1967)
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SUMMARY

A low resolution (4 A) electron density map of horse heart cytochrome c crystals has been obtained by X-ray diffraction methods. The molecule is a prolate spheroid, approximately 25 × 25 × 37 A. It has a cleft or crevice along one side into which the heme is inserted, normal to the surface, with only one of the edges of the porphyrin ring exposed to solvent. The ligands occupying coordination positions 5 and 6 of the heme iron extend out from either side of the cleft. The thioether links binding the vinyl side chains of the heme to cysteinyl residues in positions 14 and 17 of the amino acid sequence are visible. One of the iron ligands can be identified from its shape and location as the imidazole side chain of the histidyl residue in position 18. The other is not an imidazole side chain and is probably located in the carboxyterminal half of the amino acid sequence. There is little or no a-helix present, the body of the molecule being an extended chain shell around a core of packed hydrophobic side chains.

A considerable amount of knowledge is available with regard to the amino acid sequences, the activity, the physicochemical behavior, and the immunochemical properties of cytochromes c from numerous species (see Reference 1). An x-ray diffraction analysis of horse heart ferricytochrome c is in progress, and the present communication describes the position and environment of the heme group in the molecule as they have been revealed by the early stages of the analysis.

Horse heart cytochrome c, prepared by an aluminum sulfate extraction procedure (2), was crystallized in the ferric form in near saturated (NH₄)₂SO₄ containing 0.5 to 1.0 M NaCl. Two heavy atom isomorphic derivatives were prepared by diffusing K₂PtCl₄ and mercuric ion (Hg₂⁺) into crystals of deoxycholate-prepared ferricytochrome c. Both of these compounds bind to cytochrome c molecules at a single site each, and the double derivative, containing both platinum and mercury, has also been obtained. The derivatives have been characterized, the heavy atoms located, and a structure analysis in two-dimensional projection at a resolution of 4 A carried out (3).

Diffraction data for crystals of the parent protein and of the platinum and mercury derivatives have been collected in three dimensions at 4 A resolution (1475 reflections per derivative), a multiple isomorphous phase analysis has been carried out, and electron density maps have been calculated for 48 sections through the molecule, 0.86 A apart. The ratio of the mean change in F produced by the heavy atom to the mean F itself in the centrosymmetrical hk0 zone is 0.292 for platinum and 0.145 for mercury. The corresponding values over all reflections are 0.201 and 0.114, respectively. The mean figure of merit with two derivatives is 0.46, and the Kraut R factors (4) are 7.8% for platinum and 6.1% for mercury. Judging from the two-dimensional analysis (Fig. 6 of Reference 3), the low figure of merit at this point is to be attributed to the use of the bare minimum of data necessary for phase determination. However, at this stage, the individual molecules of the protein can be readily delineated and the heme group and its environment are clearly visible.

The crystals are tetragonal, space group P4₁, with one molecule of molecular weight 12,400 per asymmetrical unit (4). Cell dimensions are: a = b = 58.45 A, c (the 4-fold axis) = 42.35 A. Only 45% of the crystal by volume is protein, the rest being liquid of crystallization (5). The molecules pack in a very open manner; they are grouped most tightly around a 4-fold screw axis in the centrosymmetrical hk0 zone is 0.292 for platinum and 0.145 for mercury. The corresponding values over all reflections are 0.201 and 0.114, respectively. The mean figure of merit with two derivatives is 0.46, and the Kraut R factors (4) are 7.8% for platinum and 6.1% for mercury. Judging from the two-dimensional analysis (Fig. 6 of Reference 3), the low figure of merit at this point is to be attributed to the use of the bare minimum of data necessary for phase determination. However, even at this stage, the individual molecules of the protein can be readily delineated and the heme group and its environment are clearly visible.

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Without taking into account exterior side chains, the molecule is a prolate spheroid of dimensions roughly 25 × 25 × 37 A.
The mean electron density throughout the entire molecule is almost exactly equal to the electron density of the crystallizing medium, so that regions of more loosely packed van der Waals' interacting side chains appear negative on the electron density map. More dense regions with a high proportion of covalent bonds appear positive at this resolution. A photograph from the side with the heme crevice of a model built from contours at an electron density that would mark out the covalently bonded structures is given in Fig. 1. Fig. 3 is a similar photograph showing the metal-binding sites and an apparent "pseudo-channel" from the surface to the heme.

The center of the molecule is strongly negative, indicating most probably a region of packed hydrophobic side chains. Around this core is a dense, structured layer or shell, made up of extended polypeptide chain winding around to form an almost seamless wall with three exceptions, the heme crevice and two apparent channels to the interior. Around this shell lies a more negative region, not as negative as the innermost core and probably made up of hydrophilic side chains extending into the salt region. The salt medium around and between molecules is flat and devoid of features. In comparison with hemoglobin, myoglobin, lysozyme, and ribonuclease, cytochrome c is the smallest and devoid of features. In general agreement with recent solvent perturbation studies of Stellwagen (9). The normal to the heme plane makes an angle of 70° with the z axis, in excellent agreement with the polarized absorption spectrum results of 72 ± 3° of Eaton and Hochstrasser (10) and of 65-70° of Kabat (11). The porphyrin ring extends into the interior of the molecule, which serves as a hydrophobic pocket for the heme.

The fifth and sixth coordination ligands of the iron (H and M, Fig. 1) extend from either side of the crevice, and the thioether bonds to cysteinyl residues 14 and 17 of the amino acid sequence are visible near the top of the crevice (S and P, Fig. 1). The heme propionic acid side chains (P and M, Fig. 1) do not point out into the solvent, as they do for myoglobin and hemoglobin and as has sometimes been suggested for cytochrome c (9). Instead, they point down toward one side of the pocket, one nearer the surface than the other, perhaps associated with one or more basic side chains within the pocket. Three sections through the electron density of the heme in a direction parallel to the z axis are shown superimposed in Fig. 2B. The relationship of the prosthetic group to the molecular surface is shown in Fig. 2B.

Judging from its flattened disc shape when viewed down the z axis, its oval (y, z) cross section and its proximity to cysteinyl residues 14 and 17, the heme iron ligand marked H in Fig. 1 is the imidazole side chain of histidyl residue 18 (12). On the far side of the heme, an extended polypeptide chain runs parallel to the long axis of the molecule and bears the sixth iron ligand (M, Fig. 1). The shape of this ligand indicates it is almost certainly not a histidyl imidazole, or a tyrosyl or tryptophyl side chain. Moreover, it is not possible for the polypeptide chain, as it can be followed on the electron density maps, to extend from one side of the heme to the other in a distance short enough for the sixth ligand to arise from histidyl residues in position 26 or 33, the only other such residues in the protein (12). Evidence that only 1 histidyl residue is concerned with heme formation in cytochrome c has come from primary structure studies (13, 14). A tentative fitting of the amino acid sequence of horse heart cytochrome c (12) to the electron density contours, although necessarily of doubtful validity at the present resolution, nevertheless indicates that the sixth iron ligand most probably arises from the carboxyl-terminal half of the polypeptide chain and could readily accommodate the methionyl residue in position 80, as indicated by several lines of indirect evidence (15-21). The same tentative assignment of the polypeptide chain to the model is in remarkably good agreement with a large number of other structural implications of amino acid sequence, evolutionary, enzymic, physicochemical, and immunological data for cytochrome c.

The other features of the surface of the molecule are two "pseudo-channels" to the interior. These spaces are not empty channels in the true sense, and are not accessible to the solvent. These regions have a negative map density and are therefore regions where loosely packed side chains in the interior meet similar side chains on the exterior, without encountering the covalently bonded shell of the molecule. One such channel extends straight down from the top of the model parallel to the z axis, and the other extends inward from one side to the surface of the heme which bears the imidazole ligand, as shown in Fig. 3. The actual length of the 104-residue polypeptide chain (104 X 3.5 A) is very near to the total running chain length observed in the model. Where two segments of the chain come close to-

![Fig. 1. A model at 4 A resolution of the molecule of horse heart ferri-cytochrome c showing the regions of the covalently bonded skeleton of the molecule. The heme crevice is visible as a vertical cleft in the center of this view, with the heme group painted a darker color and seen edge on. Letters are explained in the text.](image-url)
tracing of one continuous chain becomes impossible, and the frequent
sections 13 and 12 contain the propionic acid side groups at the bottom, and section 10 shows the takeoff
directions of the two thioether links to the polypeptide chain. B, a summary
of sections 10 through 13, with an idealized heme skeleton superimposed at the same scale. The relationship of the heme to the polypeptide chain, to the bottom of its crevice and to the surface of the molecule is shown.

Fig. 2. A, four successive (y, z) sections through the heme group, with
vertical and z at 13, 12, 11 and 10/45 of a cell edge. The plane of the
heme group tilts back to the upper left. Sections 13 and 12 contain the
propionic acid side groups at the bottom, and section 10 shows the takeoff
directions of the two thioether links to the polypeptide chain. B, a summary
of sections 10 through 13, with an idealized heme skeleton superimposed at the same scale. The relationship of the heme to the polypeptide chain, to the bottom of its crevice and to the surface of the molecule is shown.

Fig. 3. A view from the right side of the molecular model of Fig. 1
showing the heavy metal-binding sites and the "pseudo-channel" to the back of the heme. The mercury site is marked by the sphere at bottom
center, and the platinum site by the one at the upper right. Note the
running lengths of density, believed to be extended polypeptide chain.
which make up the molecular surface, the nodes of intersection where the
tracing of one continuous chain becomes impossible, and the frequent
complementarity of adjacent chain shapes. If side groups were added
around these skeletal pathways, the molecular surface would show few
gaps.

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REFERENCES
1. MARGOLIASH, E., AND SCHIJTER, A., Advanc. Protein Chem., 21, 113
(1966).
2. MARGOLIASH, E., AND WALKER, E. F., in S. P. COLOWICK AND N. O.
KAPLAN (Editors), Methods in enzymology, Vol. 13, Academic
Press, New York, in press.
3. DICKERSON, R. E., KOPKA, M. L., BORDERS, C. L., VARNUM, J.,
5. DICKERSON, R. E., KOPKA, M. L., WEINZIEB, J., VARNUM, J.
AND BORDERS, C. L., in R. W. ESTATEBOOK AND T. YONETANI
Editors), Hemes and hemoproteins, Academic Press, New
(1955).
8. GEORGE, P., GLAUBER, S. C., AND SCHIJTER, A., J. Biol. Chem., 242,
1666 (1967).
192, 1125 (1961).
1621 (1966).
647 (1966).
15. HARTRY, H. A., AND CHANCE, R. W. ESTABROOK, AND T. YONETANI
Editors), Hemes and hemoproteins, Academic Press, New
Biosynthesis of Cardiolipin in *Escherichia coli*

(Received for publication, April 20, 1967)

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**SUMMARY**

Cell-free particulate fractions from *Escherichia coli* have been found to catalyze the synthesis of cardiolipin from cytidine diphosphate diglyceride and L-glycerol 3-phosphate. Evidence is presented that phosphatidylglycerol is the immediate precursor of cardiolipin in a reaction involving the transfer of a phosphatidyl moiety from CDP-diglyceride.

**Cell-free particulate enzyme preparations** (1, 2) from *Escherichia coli* catalyze the synthesis of phosphatidylglycerol from L-glycerol 3-phosphate and cytidine diphosphate diglyceride via Reactions 1 and 2 which also have been demonstrated in animal tissues (3).

L-Glycerol 3-phosphate + CDP-diglyceride $\rightarrow$ phosphatidylglycerophosphate + CMP (1)

Phosphatidylglycerophosphate $\rightarrow$ phosphatidylglycerol + Pi (2)

The bacterial enzymes catalyzing Reactions 1 and 2 have recently been separated and partially purified (2).

We now wish to report evidence that particulate enzyme fractions from *E. coli* also catalyze the further conversion of phosphatidylglycerol to cardiolipin in Reaction 3.

Phosphatidylglycerol + CDP-diglyceride $\rightarrow$ cardiolipin + CMP (3)

Cells of *E. coli* strain M1. 308 were grown on medium 63 (4) with succinate as carbon source and harvested in late log phase. The cells were disrupted in a Manton-Gaulin homogenizer, and the suspension (about 15 mg of total protein per ml) in 0.1 M Tris buffer, pH 7.4, containing 0.01 M β-mercaptoethanol was subjected to differential centrifugation at 0°C. After removal of whole cells and debris at 5,000 x g for 15 min, a particulate fraction was obtained by centrifugation at 40,000 x g for 1 hour. This particulate fraction was resuspended in a volume of the supernatant solution equal to one-fifth of the original volume and used without further fractionation.

When the enzyme was incubated with L-glycerol 3-phosphate-2-3H and CDP-diglyceride, three principal labeled lipids were formed which could be separated by chromatography on DEAE-cellulose (Fig. 1). Peak I (the first to emerge from the column) was identified as phosphatidylglycerol, and Peak III as phosphatidylglycerophosphate, by the methods of Kiyasu et al. (3) and Chang and Kennedy (2). Peak II emerged from the column in the same fractions which contained carrier bovine heart cardiolipin.

Further evidence that Peak II represents labeled cardiolipin was obtained by the following methods.

**Thin Layer Chromatography of Intact Lipid**—An aliquot of labeled lipid from Peak II (Fractions 10 + 11) was chromatographed on an Eastman-Kodak Silica Gel Sheet in the solvent system chloroform-methanol-water (65:25:4, v/v). After chromatography the water-soluble product of alkaline hydrolysis was oxidized with periodate oxidation of product of alkaline hydrolysis—The water-soluble product of alkaline hydrolysis was oxidized with

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