**Tuna Cytochrome c at 2.0 Å Resolution**

**III. COORDINATE OPTIMIZATION AND COMPARISON OF STRUCTURES**

(Received for publication, December 6, 1976)

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Optimum coordinate sets have been obtained for ferrocytochrome c and the two symmetry-independent molecules of ferricytochrome c from tuna at 2.0 Å resolution by making the best fit of models with standard bond lengths and angles to the experimental electron density maps (1977) *J. Biol. Chem.* 252, 7994–85, as a preliminary to full refinement with the experimental electron density maps (1977) *J. Biol. Chem.* 252, 7994–85. The best fit of models with standard bond lengths and angles have been fitted to the electron density maps through the building of wire models and measurement of raw coordinates. This paper also discusses the ferricytochrome and ferrocytochrome structures, including coordinate rotation to superimpose one molecule on another, and quantitative assessments of differences in atomic positions. Since real-space and phase refinement, including location of solvent peaks, will be carried out only after collection of 1.5 Å resolution parent cytochrome data (now in progress), these three papers will constitute the complete record of MIR* structure analysis at 2.0 Å resolution.

Tuna ferricytochrome c crystallizes in space group *P*4*₃*, with cell dimensions: *a* = *b* = 74.51 Å, *c* = 36.31 Å. The asymmetric unit contains two independent molecules, which will be identified as the "outer" and "inner" molecules as described in Ref. 1. Tuna ferricytochrome c crystallizes in space group *P*2₁2₁2₁ with cell dimensions: *a* = 37.32 Å, *b* = 87.02 Å, *c* = 34.51 Å, with a single molecule per asymmetric unit, to be referred to as the "reduced" molecule.

**METHODS**

*Coordinate Measurement* — Starting coordinate sets for the three independent ferri- and ferrocytochrome molecules were obtained from Kendrew wire models fitted to the MIR maps in a Richards box. Coordinates were measured from the Kendrew wire models in an electronically operated device similar to that described by Salemme and Fehr (3). In this apparatus, constructed at Caltech by Michael Ross and Robert Stroud, the model is placed on the opposite side of a vertical half-silvered mirror from a small light-emitting diode capable of being positioned by three mutually perpendicular motor-driven worm gears. The operator brings the diode into coincidence with the reflected image of an atom center, and coordinates are read out directly from potentiometer dials and recorded on paper tape. Raw coordinates are in an arbitrary coordinate frame, and must be brought into the crystallographic frame by a rotation-translation operation. The elements of the rotation matrix and translation vector are obtained by least-squares fitting to 12 reference positions along the unit cell edges in the Kendrew wire model.

This coordinate measuring device is subject to the normal errors of any Richards box — greater coordinate uncertainty in a direction perpendicular to the mirror, and distortions from mirror bending — although the latter is virtually eliminated by standing the mirror upright. In practice, we found that errors in positioning the light-emitting diode on the reflection of the atom center, approximately 0.55 mm or 0.025 Å as determined by successive remeasurements of six test amino acids, were trivial in comparison with the random small errors and distortions built into the wire model during construction. The operators were trained to recognize when the wire model was not properly positioned, and to measure coordinates in this condition.

*This work was performed with the support of National Science Foundation Grant PCM75-65586 and National Institutes of Health Grant GM-12121. This paper is Contribution 5467 from the Norman W. Church Laboratory of Chemical Biology, California Institute of Technology.*

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A large contribution of the optimization procedures described below is the removal of these mechanical inaccuracies of model building.

**Minimum Remainder** - The goal of this stage of refinement was an optimum fitting of the polypeptide chain with near standard bond parameters to the two MIR maps. It was an iterative process with three steps: visual inspection of the match between atomic positions and map density, manual adjustment of coordinates to improve that fitting, and restandardization of these perturbed coordinates with the help of either the Diamond model-building routines (4, 5), or the Chambers pseudo-energy minimization program (6).

Miniature electron density maps were drawn on one-foot square plexiglass sheets, with the thickness of plastic chosen to match the section interval so the sheets could be stacked directly atop one another without spacers. Sections of the MIR electron density maps were contoured directly onto acetate sheets by a Calcomp plotter, and the acetate was taped to the plexiglass. Atomic positions, drawn to scale on a Vectorian electrostatic printer-plotter controlled by a Data General NOVA 800 minicomputer, were recorded with alcohol-soluble ink on the back of the neighboring plexiglass sheet that was stacked against a particular acetate map section. Hence one set of coordinates could be wiped away and another set substituted without affecting the MIR maps.

Shifts were made by inspection, rotating amide planes by fitting to the carbonyl oxygen density, realigning side chains, and moving main chain into maximum density. Some effort was made to move rigid groups as a unit and to spread any shifts over several adjacent atoms, but the main purpose of the shifts was to produce an improved set of atomic guide points for the subsequent model-building step. After Diamond or Chambers model-building, minimap inspection with these rebuilt coordinates was repeated until no further improvement of map fitting could be made. We feel strongly that at this early stage of refinement, the subjective decisions that the human mind can make regarding side chain positioning and the shape of map density features are of critical importance, and cannot be replaced with ease by the most sophisticated of automatic density fitting algorithms. Only at a later stage, when the issue changes from one of correction of model errors to one of simple adjustment of a correct conformation, would we be willing to relinquish control provided by human inspection of the quality of fit.

**Orientation of Heme and Aromatic Residues** - The heme group in each of the three molecules was refined as a unit at the beginning, by fitting standard parameters as used with myoglobin to the measured heme coordinates with a rigid body least-squares superposition routine. The idealized heme group was modified only by extending the C-C bond length shown in Fig. 1 from a partially conjugated to a pure single bond value. Only the rigid framework of polyhedral ring atoms and the side chain atoms attached directly to it were used in defining heme orientation.

After iterating the heme group fitting to convergence, sections of MIR electron density were calculated parallel and perpendicular to the heme plane. These sections suggested small adjustments, so another set of coordinates could be substituted for the heme without affecting the electron density maps themselves. Unfriated with this routine, the choice was either to release the main chain from constraint and allow the program to build these residues as required for side chain/heme connectivity, or to accumulate all the errors into unrestrained side chain bond angle deformations. Neither alternative was satisfactory.

Version 4 of the Diamond real space refinement program (5) provided more flexibility. This routine can be run in two modes: deriving atomic shifts either from comparisons with the electron density map itself, or with a set of specified atomic coordinates as guide points. In the latter mode, it becomes a sophisticated model-adjusting routine, capable of moving coordinates to adjust bond lengths, bond angles, and torsion angles simultaneously toward idealized values, with relative weighting factors that are under the control of the operator. This use of the real space program will be termed the model-adjusting routine to contrast it with the simpler model-building program.

Good connections between heme and cysteine side chains could not be made with the model-building routine, but such connections for the "outer" ferriocytocrome molecule were made with ease with the model-adjusting routine, before this approach was dropped in favor of Chambers' minicomputer system.

Several physical distortions in the original wire models that occurred during construction proved to be stumbling blocks to the

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2. R. Ladner, private communication.

3. R. Diamond, personal communication.
simple model-building program. If the main chain bond angle, $\tau$, and the amide plane dihedral angle, $\omega$ (see Fig. 2), were held at their ideal values, a fitting of idealized chain to the model guide points became impossible, but if these bond angles were released from constraints, then they accumulated serious errors, as much as 30° away from expected values. These problems were cleared up by using the model-adjusting program, and allowing all three main chain bond angles, $\tau_1$, $\tau_2$, and $\tau_3$ (Fig. 2) to deviate from ideality with spring-like constraints. The best fitting with the model-building routine ended with $\tau$ values that deviated an average of 9° from ideal tetrahedral values; but iteration with the model-adjusting routine spread these distortions among all $\tau$ angles, with an average deviation of only 1.5°, and without major changes in chain conformation. The occasional serious distortions found earlier in particular $\tau$ angles were eliminated in a most satisfactory manner.

The Diamond routines were used until one of the three molecules, the outer ferricytochrome, had been taken completely through the model-adjusting program while the other two were ready for it. At this point, reasons of computing economy led us to change to a minicomputer approach as described below. It is worth emphasizing that the Diamond approach led to completely satisfactory results and worked quite well. A large central computer was required for this, however, while the Chambers routines were written for our own laboratory minicomputer, a Data General NOVA 800. This economic incentive was one factor in a decision to change, but another was the fact that it was considerably less difficult for a novice to learn to use the Chambers programs.

Chambers Pseudo-energy Minimization—For the reasons just...
given, fitting of coordinates to the MIR electron density map was continued using a different constraints program written by Chambers and Stroud (6) for the Data General NOVA 800 minicomputer equipped with 32,000 sixteen-bit words of core, a 2.5 megabyte disk, magnetic tape, and an electrostatic matrix printer-plotter. This program reduces the deviations from standard bond lengths, bond angles, dihedral angles, and initial atom positions (a necessary damping factor) by using a method of steepest descents to minimize the sum of squares of all of these deviations simultaneously. Constrained shifts are applied one amino acid residue at a time in a "zone refinement" procedure, with the carbonyl group preceding and the amide nitrogen atom following the residue included as boundary conditions to ensure continuity between residues. In operation, the terms representing deviations of the different coordinates from the original set are given unit weights, whereas terms representing deviations from standard bond parameters are given increasing weight with each pass through the atom list, thus accelerating convergence. The ideal bond parameters used were those of Marsh and Donohue (7).

An important feature of the pseudo-energy minimization routine is the ability to assign different relative weights to bond length and bond angle discrepancies, since bond and dihedral angles may vary significantly from ideality (8-10), whereas bond lengths are less likely to do so. Two or three passes through the atom list usually were sufficient to ensure convergence, with the bond length terms being weighted three times as much as bond and dihedral angle terms for the first two passes, and six times as much thereafter.

Phenylalanine, tyrosine, histidine, and tryptophan residues were given special treatment, since these planar residues were so well defined in the electron density maps, they were positioned by inspection of parallel and perpendicular sections as described earlier and then held immobile during refinement. Atoms such as these which were not to be moved by the Chambers program were indicated by inserting them in the atom list after the carbonyl group of the

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Fig. 4. Main chain stereo drawings of (upper) outer, (middle) inner, and (lower) reduced cytochrome c molecules viewed from the right side. Notice the impression of emptiness below and to the right of the heme group caused by the omission of the interior side chains that in the full molecule are packed around the heme.
residue to which they belonged, but before the amide nitrogen of the next residue.

Instead of building ideal residues with standard parameters as the Diamond model-building program does, pseudo-energy minimization only demands a minimization of deviations from standard bond parameters and starting coordinates, with the relative importance of these factors under the control of the operator. This feature allowed for easy application of constraints to the heme iron connections at histidine 18 and methionine 80. The N—Fe—S angle was constrained at 180° and the N—Fe—heme plane angle at 90°, forcing the S—Fe—heme plane angle also to remain at 90°. It was our opinion that any deviations from ideal values at the stage of 2 Å resolution MIR maps could not possibly be reliable, and that such deviations, if they exist, can best be seen at the 1.5 Å stage of refinement. The two cysteine heme connections also could be handled in a straightforward way. Atoms C, and C, (Fig. 1) were held fixed along with the rest of the heme, the sulfur atom was constrained to a cone centered on the C,—C, axis, and the methyl-C, was kept tetrahedral to C, and S.

The logistics of use of the Chambers routine were similar to that for the Diamond programs. After each run to convergence through the constraints program, new coordinates were plotted on the appropriate minimap. Manual shifts were made in atomic positions to bring them into better coincidence with map density, and these adjusted coordinates then were subjected to constrained pseudo-energy minimization again. In all, four such alternations of map examination and coordinate adjustment were carried out on each of the three molecules, requiring a grand total of 4 h of refinement and 9 h of plotting time on the NOVA 800 minicomputer.

Rigid Body Superposition — The rigid body superposition routine brings two molecules to coincidence by rotating them to minimize the sum of squares of distances between corresponding atomic positions, assuming that the center of mass of each molecule lies at the common origin of coordinates. The solution for the rotation matrix
| Residue | Amino Acid | Outer (O) | Inner (I) | Reduced (R) | Δ: O/I | O/R | I/R |
|---------|------------|-----------|-----------|-------------|--------|-----|-----|
|         |            | φ         | ψ         | φ           | ψ      |     |     |
| 1       | Gly        | -108      | 91        | 121        | -56    | -99 | -14 |
| 2       | Asp        | -11       | 85        | 135        | -94    | -92 | -69 |
| 3       | Val        | -50       | -23       | -43        | -70    | -42 | -77 |
| 4       | Ala        | -70       | -57       | -41        | -59    | -50 | -49 |
| 5       | Lys        | -63       | -21       | -55        | -31    | -51 | -55 |
| 6       | Gly        | -65       | -44       | -70        | -44    | -47 | -60 |
| 7       | Lys        | -64       | -45       | -58        | -65    | -54 | -61 |
| 8       | Lys        | -53       | -57       | -42        | -61    | -50 | -72 |
| 9       | Thr        | -69       | -28       | -65        | -45    | -27 | -60 |
| 10      | Phe        | -65       | -45       | -55        | -45    | -30 | -86 |
| 11      | Val        | -61       | -28       | -48        | -66    | -31 | -51 |
| 12      | Gin        | -48       | -82       | -60        | -30    | -57 | -54 |
| 13      | Lys        | -104      | 13        | -110       | -30    | -96 | -48 |
| 14      | Cys        | -111      | 55        | -107       | -19    | -92 | -33 |
| 15      | Ala        | -43       | -42       | -75        | -2     | -33 | -37 |
| 16      | Gin        | -43       | -45       | -88        | -8     | -78 | -15 |
| 17      | Cys        | -102      | -8        | -138       | -11    | -113 | -12 |
| 18      | His        | -143      | 166       | -146       | 160    | -145 | 161 |
| 19      | Thr        | -137      | 153       | -115       | 158    | -120 | 117 |
| 20      | Val        | -121      | 2         | -113       | -34    | -107 | 71  |
| 21      | Glu        | -85       | 154       | -63        | 153    | -178 | 138 |
| 22      | Asn        | -61       | 134       | -56        | 122    | -39  | 102 |
| 23      | Gly        | 79        | -2        | 60         | -7     | 114  | 26  |
| 24      | Lys        | -47       | 152       | -43        | 161    | -38  | 112 |
| 25      | His        | -71       | 141       | -13        | 121    | -37  | 186 |
| 26      | His        | -96       | 176       | -94        | 134    | -45  | 166 |
| 27      | Lys        | -95       | -117      | -120       | -131   | -180 | -125 |
| 28      | Val        | -80       | -41       | -65        | -53    | -77  | 46  |
| 29      | Gly        | -119      | 175       | -98        | 161    | -99  | 178 |
| 30      | Pro        | -68       | 168       | -73        | 173    | -85  | 180 |
| 31      | Asn        | -82       | 131       | -79        | 140    | -83  | 156 |
| 32      | Leu        | -89       | 8         | 96         | -12    | -190 | 5  |
| 33      | Trp        | -51       | 115       | -44        | 139    | -45  | 98  |
| 34      | Gly        | -47       | -5        | 68         | 9      | 116  | 23  |
| 35      | Leu        | -32       | 66        | -52        | -40    | -10  | 65  |
| 36      | Phe        | -35       | 132       | -60        | 125    | -47  | 98  |
| 37      | Gly        | 82        | -23       | 75         | 8      | 125  | -23 |
| 38      | Arg        | -83       | 163       | -97        | 167    | -106 | 169 |
| 39      | Lys        | -86       | 129       | -79        | 131    | -72  | 174 |
| 40      | Thr        | -42       | 140       | -44        | 126    | -62  | 111 |
| 41      | Gly        | 86        | 5         | 89         | 75     | 95   | -74 |
| 42      | Gin        | -83       | -40       | -121       | 0      | 25   | -88 |
| 43      | Ala        | -35       | 89        | -40        | -171   | 29   | 100 |
| 44      | Glu        | -39       | 160       | -104       | 128    | -77  | 128 |
| 45      | Gly        | 73        | -13       | 90         | -117   | 95   | -98 |
| 46      | Tyr        | -98       | 139       | -13       | 131    | -14  | 128 |
| 47      | Ser        | -92       | 95        | -88        | 96     | -89  | 82  |
| 48      | Tyr        | -83       | 175       | -84        | 163    | -66  | -176 |
| 49      | Thr        | -101      | 122       | -93        | 127    | -83  | 150 |
| 50      | Asp        | -24       | -44       | -42        | -32    | -66  | -33 |
| 51      | Ala        | -50       | -51       | -67        | -44    | -50  | -62 |
| 52      | Asn        | -54       | 39        | -53        | -37    | -59  | -57 |
| 53      | Lys        | 72        | 36        | 90         | 26     | 51   | 14  |
| 54      | Ser        | -95       | -25       | -76        | -34    | -130 | -38 |
| 55      | Lys        | -47       | -36       | -22        | -66    | -1   | -122 |
| 56      | Gly        | 55        | 31        | 100        | -8     | 173  | 53  |
| 57      | Ile        | -90       | 175       | -92        | 162    | -160 | 169 |
| 58      | Val        | -99       | 127       | -101       | 145    | -119 | 151 |
| 59      | Trp        | -67       | 125       | -74        | 109    | -87  | 130 |
| 60      | Asn        | -161      | 169       | -137       | 173    | -128 | 159 |
where $M$ is the $3 \times 3$ least squares solution of Equation 1, $B'$ is the just defined is a linear transformation matrix rather than a rotation transpose of $B$, and the superscript $-1$ represents the inverse. $M$ as constraint, which is imposed by iterating:

$$M = ABT(BB')^{-1}$$

where the approximation to total equality arises from inherent discrepancies between the molecules, minimized by the least squares process. Diamond has shown that the desired solution to Equation 1 is:

$$M = ABT(BB')^{-1}$$

where $M$ is the $3 \times 3$ least squares solution of Equation 1, $B'$ is the transpose of $B$, and the superscript $-1$ represents the inverse. $M$ as just defined is a linear transformation matrix rather than a rotation matrix, since neither Equations 1 nor 2 impose the orthogonality constraint, which is imposed by iterating:

$$2M_{i,j} = M_{i,j} + (M^T)^{-1}$$

The principal advantage of this approach is that no initial guess for the orientation matrix is required, while the main disadvantage is that molecule $b$ cannot be planar. In heme superposition, planarity of the heme was circumvented by including the sulfur atom of the idealized heme to guide coordinates taken from the electron density maps, and also to make the detailed comparisons between coordinate sets for whole molecules that will be described later.

**RESULTS**

The cytochrome coordinates resulting from this optimization process are available either on microfiche* or on magnetic pages. Orders should specify the title, authors, and reference to this publication. A computer program written in this laboratory with the above algorithm was used to fit an idealized heme to guide coordinates taken from the electron density maps, and also to make the detailed comparisons between coordinate sets for whole molecules that will be described later.

* Atomic coordinates and the data used to prepare Figs. 12 and 13 are given below is adapted from Diamond (4). The two $N$-atom molecules are designated as $a$ and $b$. Let $A$ be a $3 \times N$ matrix with each column of $A$ being the coordinates of one of the atoms of molecule $a$, and let $B$ be similarly defined. Then $R$ is the desired $3 \times 3$ rotation matrix that superposes molecules $a$ and $b$:

$$A = RR$$

where the approximation to total equality arises from inherent discrepancies between the molecules, minimized by the least squares process. Diamond has shown that the desired solution to Equation 1 is:

$$M = ABT(BB')^{-1}$$

where $M$ is the $3 \times 3$ least squares solution of Equation 1, $B'$ is the transpose of $B$, and the superscript $-1$ represents the inverse. $M$ as just defined is a linear transformation matrix rather than a rotation matrix, since neither Equations 1 nor 2 impose the orthogonality constraint, which is imposed by iterating.

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FIG. 6. Ramachandran plot of α carbon conformations in the outer ferricytochrome c molecule. Glycine residues, shown by open circles, are identified by residue number; other residues are indicated by black dots. The contour level drawn is at 3 kcal/mol above the global minimum of energy according to Pullman’s revised quantum calculations for alanine (11). All glycines in the region around (90°, 0°) except residue 56 are participants in $\beta$$_{13}$ bends of type II.

Main Chain Conformation and Ramachandran Plots—Stereo computer drawings of the main chain atoms for the outer, inner, and reduced molecules are shown in three standard orientations: front view in Fig. 3, right side in Fig. 4, and top in Fig. 5. The chain folding in general is the same in all three molecules with specific exceptions to be discussed later. Comparison of the three molecules is facilitated by the Ramachandran angles ($\phi$, $\psi$) given in Table II. These angles in turn are displayed in Ramachandran plots in Figs. 6 through 8, along with a contour level representing an energy of 3 kcal/mol above the global minimum according to the refined alanyl calculations of Maigret et al. (11). With other proteins as well as with cytochrome c, this 3 kcal boundary has proven to be a better summary of actual observed conformation than have the classical hard sphere Ramachandran boundaries (12). The hard sphere and quantum calculation boundaries are compared in Fig. 9, which also contains the idealized conformation positions for several common forms of secondary structure.

The physical meaning of the hard sphere boundaries is best understood with the help of the “derivation diagram” in Fig. 10 showing how a Ramachandran plot is built up from individual steric contacts, and the definitions of atom numbering in Fig. 11. If no R group side chain were present (as in glycine), then the conformation region forbidden because of steric clash or overlap of atoms would be the roughly cross-shaped zone through the center of Fig. 10. The atomic overlaps that occur when this boundary is traversed from one of the four allowed corner regions are indicated on the forbidden side of each segment of the boundary. The center of the diagram corresponds to the most severe clash between oxygen $O_1$ and hydrogen $H_2$. The midpoint of the horizontal axis is the maximum overlap between carbonyl oxygens, $O_1$—$O_2$, and the center of the vertical axis is the worst clash between amide hydrogens, $H_1$—$H_2$.

This polyglycine diagram is limited even more when side
Fig. 9. Comparison of Ramachandran hard sphere allowed regions (dashed lines) with Pullman quantum energy levels at 0 kcal and 2 kcal/mol above the global minimum (solid lines). Several ideal conformation positions are shown: $A = \text{antiparallel } \beta \text{ sheet}, P = \text{parallel } \beta \text{ sheet}, \alpha = \text{right-handed } \alpha \text{ helix}, L = \text{left-handed } \alpha \text{ helix}, \beta = 3_{10} \text{ helix}, I = 3_{10} \text{ turn of type I, II = 3}_{10} \text{ bend of type II.}$

Fig. 10. "Derivation diagram" for the Ramachandran plot. Boundaries are drawn between allowed and forbidden regions using a hard sphere model, and the clashing atoms are identified on the forbidden side of the boundary. (See Fig. 11 for atom numbering.) Only four allowed zones are found: the $\beta$ sheet region at the upper left, the right-handed $\alpha$ helix zone below it, the left-handed $\alpha$ helix rectangle identified by $L$, and a negligible triangle above it.

The following chains, $R$, are present. Configurations with $\phi$ between 65° and 175° are disallowed for all $\psi$ because of overlap between $O_1$ and the side chain; and all configurations with $\psi$ between $-70°$ and $-170°$ are eliminated because of clash between $H_2$ and the side chain. These vertical and horizontal forbidden belts leave only three allowed regions in the hard sphere plot as outlined in heavy black in Fig. 10: the right- and left-handed helices, containing points $\alpha$ and $L$, and the $\beta$ sheet zone at upper left, containing $A$ and $P$.

A comparison of these limits with Pullman's energy contours (Fig. 9) shows three principal discrepancies. The left-handed $\alpha$ helix is not favored in the quantum calculations, but a small region to its lower right is connected to the $\beta$ sheet region by a tenuous energy valley. The right-handed $\alpha$ helix region, in turn, is elongated to the lower right of the hard sphere boundary. Most significant of all, the classically forbidden zone between the $\alpha$ helix and the $\beta$ sheet regions is energetically allowed by the quantum calculations.

Examination of the observed cytochrome plots in Figs. 6 through 8 shows that for all three of these regions, the quantum limits are better than the hard sphere boundaries. This is a confirmation of earlier experience reported with seven other globular proteins (13). Comparison of Figs. 9 and 10 reveals which of the hard sphere contacts are "softer" than expected. Both of the contacts between an atom connected directly to the $\alpha$ carbon and the extreme atom on the other amide group ($N_1$ against $H_2$, and $C_\gamma$ against $O_1$) appear to be relatively soft, leading to the diagonal energy valleys through both the right- and left-handed $\alpha$ helix positions. As Ramachandran has observed (12), these clashes can be relieved by opening up the $N_1-C_\gamma-C$ angle slightly beyond its ideal 109.5° value. Space-filling models reveal that both the $N_1-H_2$ and $C_\gamma-O_1$ clashes are grazing contacts, which are eliminated by a relatively small increase in this $C_\gamma$ angle. The $N_1-H_2$ clash valley connecting $\alpha$ helices and $\beta$ sheets is well populated in cytochrome $c$ and other proteins (14), but the small local minimum around (100°, $-30°$) is populated only by glycines, mainly those involved in $\beta$ or $3_{10}$ bends$^6$ of type II.

Outer, inner, and reduced molecules were deliberately treated independently during model building and coordinate optimization, with no attempt made to impose the same chain conformation. This was done with the intention of using outer versus inner comparisons as a control to estimate the significance of differences observed between both of these and the reduced molecule. The scatter of labeled glycine positions in Figs. 6 to 8 furnishes one measure of the conformational differences between molecules, and the columns marked by $\Delta$

Charlotte Schellman has pointed out the growing confusion in the literature between the term "$\beta$ turn" and the older "$\beta$ sheet", the two being structurally quite different. The so-called "$\beta$ turn" has a hydrogen bonding pattern like one turn of a $3_{10}$ helix, and has already been designated as a "$3_{10}$ bend" in the literature (2, 14). To avoid future confusion, we shall use the nomenclature "$3_{10}$ bend" exclusively.

Fig. 11. Numbering scheme for amide atoms used in the derivation diagram and the text. The conformation shown here is designated as (180°, 180°) and appears at the four corners of the Ramachandran diagram.
in Table II provide another. These $\Delta$ values are the distances in degrees between points representing the same amino acid residue in any two of the three Ramachandran plots, calculated as indicated in the legend to the table. As would be expected from the physical constraints of model building, disagreement between the three molecules is least in regions of a helix. For 44 residues with $\alpha$ helical Ramachandran angles, the mean discrepancies were $22^\circ$ for outer versus inner, $26^\circ$ for outer versus reduced, and $23^\circ$ for inner versus reduced. For 54 nonhelical residues the discrepancies were somewhat larger: $32^\circ$ for outer/inner, $43^\circ$ for outer/reduced, and $44^\circ$ for inner/reduced. These numbers tell us that the reduced molecule is slightly less like the other two in the nonhelical regions of the chain, but they cannot indicate a priori whether these differences are real or are artifacts of the maps and the model-building process. This point will be re-examined under "Discussion."

Rotation and Superposition Comparisons—As a further comparison of the three molecules, coordinates of the inner and reduced molecules were rotated into the coordinate frame of the outer molecule, using the least squares routine described under "Methods." Atoms used to obtain the transformation matrices were the heme iron and all $\alpha$ carbon atoms from residues 4 through 100, omitting residues at both ends where conformational differences might be expected. Coefficients of the final transformation matrices are given in Table III.

After rotation of all three molecules into the same coordinate frame, several pairwise comparisons of conformation were made. Fig. 12 shows the mean differences in equivalent atom positions between two molecules as a function of residue number, using only those atoms rigidly attached to the main chain: N, C$,\alpha$, C$,\beta$, and carbonyl C and O. Fig. 13 is a similar plot showing mean differences in atomic positions for side chain atoms beyond C$,\beta$. For main chain atoms the mean difference in atomic positions averaged over all residues was 0.81 Å when comparing outer and inner molecules, 1.01 Å for outer versus reduced, and 1.04 Å for inner versus reduced. These figures establish an error level against which other comparisons should be judged. As would be expected, the side chains show more variation in position from one molecule to another (Table IV). Internal side chain atoms vary in position by an average of 1.0 to 1.3 Å, again probably representing inaccuracies in map interpretation and model-building, but the external side chains vary from 1.7 Å to 2.2 Å from one molecular comparison to another. In all of the comparisons of Table IV, the two ferricytochrome molecules are slightly more alike than either is like the reduced molecule, but the differences are so small that one hesitates to see in them a real difference in conformation between oxidation states. The actual numbers used in constructing Figs. 12 and 13 and Table IV are available on microfiche. A more informative comparison of main chain pathways is provided by Figs. 14 and 15 and Table V. For pairwise comparisons of molecules in a common coordinate frame, Fig. 14 shows the distance between centers of corresponding amide planes and Fig. 15 the angles between amide plane normals. This way of comparing chain configurations is the easiest to use with the stereo drawings of Figs. 3 to 5. Over all residues the average distance between corresponding amide plane centers is 0.74 Å for outer versus inner, 0.89 Å for outer versus

![Fig. 12. Rotation comparison of three cytochrome c molecules. Plots of mean distance between equivalent atoms in the main chain in Angstroms as a function of residue number. In each case, the atoms included in the comparison are main chain N, C$,\alpha$, C$,\beta$, and the carbonyl O. I, 2, 3, etc. indicate residue numbers 10, 20, 30, etc.](http://www.jbc.org/)

![Downloaded from http://www.jbc.org/](http://www.jbc.org/)
Cytochrome c Coordinates at 2.0 Å

Fig. 13. Plots of mean distance between equivalent atoms beyond Cα in the side chains in Angstroms as a function of residue number. Surface and external side chains are plotted up from the zero line, and internal, buried side chains are plotted down to differentiate them. I, 2, 3 represent positions 10, 20, 30 along the chain.

| Atoms being compared                  | Outer versus inner | Outer versus reduced | Inner versus reduced |
|---------------------------------------|--------------------|----------------------|----------------------|
| Main chain: N, Cα, C                  | 0.77               | 0.91                 | 0.96                 |
| Main chain: N, Cα, Cβ, C, O           | 0.81               | 1.01                 | 1.04                 |
| Side chains beyond Cβ                 | 1.54               | 1.84                 | 2.03                 |
| Internal side chains only             | 1.03               | 1.33                 | 1.30                 |
| External side chains                  | 1.69               | 1.99                 | 2.24                 |

The mean difference (arithmetic mean, not root mean square) in positions of main chain atoms is 1.0 Å or less in all three two-molecule comparisons (Table IV). Individual amino acid residues exceed twice this value only at the two ends of the chain where conformational differences are expected, and at residues 86 and 87, where the reduced molecule differs from the other two (Figs. 12 and 14, Table V). The histograms of Figs. 12 and 14, and the averages in Table IV suggest that the inner and outer molecules are slightly more like one another than either is like the reduced molecule, but the effect is so small that it probably is attributable to the slightly better quality of the ferricytochrome data rather than to any real oxidation state conformational differences.

The orientation of the polypeptide chain along this common path shows a few more differences, as can be seen from Fig. 15 and Tables II and V, and verified in the stereo drawings of Figs. 3 to 5. In addition to expected differences at both ends of the polypeptide chain, the angles between amide plane normals (Fig. 15 and Table V) and the distances between Ramachandran plot points (Table II) exceed 90° at just four places in the molecules: residues 20-21, 41-45, 55-56, and 86-87. The orientation of the amide plane following position 20 differs in

Table IV

Mean differences in atomic positions in three cytochrome molecules

reduced, and 0.94 Å for inner versus reduced. The corresponding average angles between amide plane normals are 20°, 28°, and 28°.

DISCUSSION

Main Chain Conformation - The stereo drawings of Figs. 3 to 5, Tables II and V, and the histograms of Figs. 12, 14, and 15 provide several means of comparing main chain differences in the three molecules.

The path followed in each case is effectively the same. The
The reduced model differed from the other two. On the bottom of the molecule, the reduced molecule was built differently at amides 41 and 42, all three differed at 43, and the outer molecule was different at position 45. It was difficult to build the model through the electron density in this region of the ferrocytochrome map, and most of the conformational discrepancies probably should be ascribed to the reduced model. At positions 55 and 56 the reduced model again is slightly different from the other two. In the last region, the outer molecule differs at position 86 and the reduced at 87. In no case is the inner molecule the odd conformation of the three.

Some of the trouble just mentioned can be ascribed to the glycines at positions 41, 45, and 56. As a general rule, the lack of a visible side chain branching from a glycyl \( \alpha \) carbon made the fitting of the amide planes on either side more difficult. For all three molecular comparisons (Table V), the mean angle discrepancy between amide plane normals was 25.3°. But this figure was 30.7° for amide planes on either side of glycine \( \alpha \) carbons, to be compared with 23.9° in nonglycine, nonhelical chain, and only 15.3° in the more constrained \( \alpha \) helices.

All of these amide plane conformational differences are visible in the stereo drawings of Figs. 3 to 5, but do they represent true structure differences, as opposed to simple ambiguities in chain-fitting to electron density? In our opinion, they do not. The outer molecule differs from the other two (residues 43, 45, and 86) nearly as often as the reduced molecule does (residues 20, 41-43, 55-56, and 87), whereas one would not expect to find real differences in main chain conformation between the two oxidized cytochrome molecules. If the outer and inner molecule comparisons are taken as a control, then the differences between these and the reduced molecule are not significant. Instead, the observed differences among all three are exactly what would be expected from ambiguities.
in map fitting: a correct overall chain path traced through the center of gravity of electron density, but occasional uncertainty in amide plane rotation along this chain. At 2.0 Å resolution, there is no unambiguous difference in main chain conformation among the three molecules. The inner molecule, which always agrees with at least one of the other two, probably is the most accurate representation of the cytochrome c main chain conformation.

**Side Chain Conformations**—The mean differences between corresponding atoms in side chains beyond the β carbon atom are plotted as a function of residue number in Fig. 13. Bars for external side chains extend up from the baseline and those for internal side chains extend down, so the two classes can be easily distinguished. One would expect external side chains to show greater variability in position among the three molecules, and there is no reason to assume that these differences are not real. Two examples that are of interest because of their evolutionary invariance among eukaryotes and their proximity to the heme are lysine 79 and arginine 38. The side chain of lysine 79 has density suggesting a hydrogen bond to the main chain carbonyl group of residue 47 in both oxidized molecules, but this connection appears to be broken in the reduced protein (Fig. 16). Unfortunately, this is an intermolecular contact surface in the reduced molecule, with lysine 79 pushed against glutamic acid in a manner that suggests an interaction of its positive charge with the buried propionic acid group (Fig. 17). Its density is well defined in both the outer and inner molecules. In the reduced map, it is ill defined and has been built out into the solvent in the way that can be seen from the stereo drawings. About all that can be said from this map is that no density is observed along the molecular surface corresponding to that in the oxidized maps, so the change probably is real. This is not an intermolecular contact surface in either crystal form.

Internal, buried side chains would be less likely to adopt different conformations in the two ferricytochrome molecules. Differences observed between the outer and inner molecule therefore should furnish a calibration of map quality, and a guide to the believability of changes seen between the oxidized and reduced molecules. Ring-containing side chains were fitted carefully to map density using plane and cross-sections as described earlier. The results are summarized in Table VI, giving the distances between ring centers and angles between ring plane normals in pairwise comparisons of the three molecules. Mean displacements are 0.60 Å between the two oxidized molecules, and 0.85 Å and 1.01 Å between an oxidized and a reduced molecule. The largest displacements are 1.94 Å between tryptophan 33 in the inner and reduced molecules, 1.83 Å between phenylalanines 10 in the same two molecules, and 1.65 Å between tryptophans 59 in the outer and reduced molecules. The tryptophan 33 shift may possibly be real; the reduced molecule is packed against a neighbor related to it by a 2-fold screw axis parallel to x (approximately the vertical axis in Fig. 16), so the conformational difference probably is induced by crystal packing.

Arginine 38 appears to lie flat against the surface of the oxidized molecules in a manner that suggests an interaction of its positive charge with the buried propionic acid group (Fig. 17). Its density is well defined in both the outer and inner molecules. In the reduced map, it is ill defined and has been built out into the solvent in the way that can be seen from the stereo drawings. About all that can be said from this map is that no density is observed along the molecular surface corresponding to that in the oxidized maps, so the change probably is real. This is not an intermolecular contact surface in either crystal form.

**TABLE V**

| AA | 1/E | 1/IP | 1/FP | 1/D | 1/EP | 1/FP |
|----|-----|------|------|-----|------|------|
| 1  |     |      |      |     |      |      |
| 2  |     |      |      |     |      |      |
| 3  |     |      |      |     |      |      |
| 4  |     |      |      |     |      |      |
| 5  |     |      |      |     |      |      |
| 6  |     |      |      |     |      |      |
| 7  |     |      |      |     |      |      |
| 8  |     |      |      |     |      |      |
| 9  |     |      |      |     |      |      |
| 10 |     |      |      |     |      |      |
| 11 |     |      |      |     |      |      |
| 12 |     |      |      |     |      |      |
| 13 |     |      |      |     |      |      |
| 14 |     |      |      |     |      |      |
| 15 |     |      |      |     |      |      |
| 16 |     |      |      |     |      |      |
| 17 |     |      |      |     |      |      |
| 18 |     |      |      |     |      |      |
| 19 |     |      |      |     |      |      |
| 20 |     |      |      |     |      |      |
| 21 |     |      |      |     |      |      |
| 22 |     |      |      |     |      |      |
| 23 |     |      |      |     |      |      |
| 24 |     |      |      |     |      |      |
| 25 |     |      |      |     |      |      |
| 26 |     |      |      |     |      |      |
| 27 |     |      |      |     |      |      |
| 28 |     |      |      |     |      |      |
| 29 |     |      |      |     |      |      |
| 30 |     |      |      |     |      |      |
| 31 |     |      |      |     |      |      |
| 32 |     |      |      |     |      |      |
| 33 |     |      |      |     |      |      |
| 34 |     |      |      |     |      |      |
| 35 |     |      |      |     |      |      |
| 36 |     |      |      |     |      |      |
| 37 |     |      |      |     |      |      |
| 38 |     |      |      |     |      |      |
| 39 |     |      |      |     |      |      |
| 40 |     |      |      |     |      |      |
| 41 |     |      |      |     |      |      |
| 42 |     |      |      |     |      |      |
| 43 |     |      |      |     |      |      |
| 44 |     |      |      |     |      |      |
| 45 |     |      |      |     |      |      |
| 46 |     |      |      |     |      |      |
| 47 |     |      |      |     |      |      |
| 48 |     |      |      |     |      |      |
| 49 |     |      |      |     |      |      |
| 50 |     |      |      |     |      |      |
| 51 |     |      |      |     |      |      |

**TABLE VI**

**Comparisons of amide plane orientations**

Distances (in Angstroms) are measured between equivalent amide plane centers in two molecules rotated for maximum overlap as described in the text. Plane normal angles (in degrees) are angles between normal vectors to the amide planes. AA = amino acid residue number; O = outer molecule; I = inner molecule; R = reduced molecule.

| AA | 1/E | 1/IP | 1/FP | 1/D | 1/EP | 1/FP |
|----|-----|------|------|-----|------|------|
| 1  |     |      |      |     |      |      |
| 2  |     |      |      |     |      |      |
| 3  |     |      |      |     |      |      |
| 4  |     |      |      |     |      |      |
| 5  |     |      |      |     |      |      |
| 6  |     |      |      |     |      |      |
| 7  |     |      |      |     |      |      |
| 8  |     |      |      |     |      |      |
| 9  |     |      |      |     |      |      |
| 10 |     |      |      |     |      |      |
| 11 |     |      |      |     |      |      |
| 12 |     |      |      |     |      |      |
| 13 |     |      |      |     |      |      |
| 14 |     |      |      |     |      |      |
| 15 |     |      |      |     |      |      |
| 16 |     |      |      |     |      |      |
| 17 |     |      |      |     |      |      |
| 18 |     |      |      |     |      |      |
| 19 |     |      |      |     |      |      |
| 20 |     |      |      |     |      |      |
| 21 |     |      |      |     |      |      |
| 22 |     |      |      |     |      |      |
| 23 |     |      |      |     |      |      |
| 24 |     |      |      |     |      |      |
| 25 |     |      |      |     |      |      |
| 26 |     |      |      |     |      |      |
| 27 |     |      |      |     |      |      |
| 28 |     |      |      |     |      |      |
| 29 |     |      |      |     |      |      |
| 30 |     |      |      |     |      |      |
| 31 |     |      |      |     |      |      |
| 32 |     |      |      |     |      |      |
| 33 |     |      |      |     |      |      |
| 34 |     |      |      |     |      |      |
| 35 |     |      |      |     |      |      |
| 36 |     |      |      |     |      |      |
| 37 |     |      |      |     |      |      |
| 38 |     |      |      |     |      |      |
| 39 |     |      |      |     |      |      |
| 40 |     |      |      |     |      |      |
| 41 |     |      |      |     |      |      |
| 42 |     |      |      |     |      |      |
| 43 |     |      |      |     |      |      |
| 44 |     |      |      |     |      |      |
| 45 |     |      |      |     |      |      |
| 46 |     |      |      |     |      |      |
| 47 |     |      |      |     |      |      |
| 48 |     |      |      |     |      |      |
| 49 |     |      |      |     |      |      |
| 50 |     |      |      |     |      |      |
| 51 |     |      |      |     |      |      |
The normals to the different ring planes also differ only by small amounts from one molecule to the next. The largest rotations are $36.5\degree$ between histidines 26 in the outer and inner molecules, $26.6\degree$ between these residues in inner and reduced, $25.5\degree$ between prolines 71 in inner and reduced, and $24.9\degree$ between tyrosines 74 in outer and reduced. The histidine 26 discrepancies clearly reflect disorder in the position of this residue as seen in the electron density maps. By Table VI, histidines 18 have nearly the same orientation in the three molecules, and the cross-sections in Fig. 19 (a to c) show that the map density for these rings is quite flat and well defined. In contrast, the cross-sections through histidines 26 (Fig. 19,
Fig. 17. Right side view of cytochrome molecules showing all side chains. Upper, outer; middle, inner; and lower, reduced molecules.

(d to f) indicate an appreciable amount of rotational disorder around the C-C$_2$ bond axis. Histidine 18 is ligated to the iron atom inside the molecule, whereas histidine 26 is only hydrogen bonded to the main chain carbonyl oxygen of residue 44 on the molecular surface, so their observed relative order and disorder are understandable. The contrasting numbers in Table VI for residues 18 and 26 probably are structurally meaningful. The other discrepancies in this table are not as dramatic, but their magnitudes correlate generally with the appearance of the cross-sections for residues shown in Refs. 1 and 2. At this resolution, most of the discrepancies in Table VI are explained by the degree of definition of rings in the electron
density maps. None of the rings can be said to have clearly defined, different, orientations in the three molecules.

Internal leucines and isoleucines (residues 32, 35, 64, 68, 85, 94, and 98) generally have similar conformations in the three molecules, and the differences are distributed among the three models in such a way as to suggest that they are model-fitting uncertainties and not genuine differences in structure. Umbrella inversions of conformation at the γ carbon in leucines occur in several cases, and are often difficult to choose between from the electron density maps.

As perhaps the most stringent test of the similarity of the three molecules, divorced from any possible errors of interpretation and model-building, two difference maps were calculated, in which the inner or reduced molecules, respectively, were rotated into coincidence with the outer molecule, and the actual electron densities were subtracted to produce outer minus inner and outer minus reduced difference maps. Although the heme and α carbon positions were used to establish the rotation parameters (Table III), no other structural interpretations contributed to these two difference maps. In regions beyond the boundaries of the molecules being compared, the difference maps were full of large positive and negative peaks.
**Cytochrome c Coordinates at 2.0 Å**

| Residue | Amino acid | Distances | Plane normals |
|---------|------------|-----------|---------------|
|         |            | O/I  | O/R  | I/R  | O/I  | O/R  | I/R  |
| 10      | Phe        | 0.50 | 1.35 | 1.83 | 9.4° | 14.6° | 11.6° |
| 18      | His        | 0.57 | 0.71 | 0.68 | 6.7  | 4.6   | 2.8   |
| 26      | His        | 0.81 | 0.60 | 0.53 | 36.5 | 10.0  | 26.6  |
| 30      | Pro        | 0.25 | 0.54 | 0.42 | 11.8 | 18.5  | 18.1  |
| 33      | Trp        | 0.97 | 1.06 | 1.94 | 10.2 | 6.3   | 15.9  |
| 36      | Phe        | 0.86 | 0.53 | 0.82 | 12.8 | 14.9  | 14.1  |
| 46      | Tyr        | 0.57 | 0.11 | 0.69 | 7.0  | 16.7  | 22.8  |
| 48      | Tyr        | 0.23 | 0.31 | 0.50 | 4.2  | 20.5  | 17.4  |
| 59      | Trp        | 0.51 | 1.65 | 1.40 | 12.9 | 15.2  | 5.6   |
| 67      | Tyr        | 1.00 | 1.28 | 1.56 | 12.5 | 22.4  | 11.0  |
| 71      | Pro        | 0.49 | 1.15 | 1.31 | 9.7  | 17.3  | 25.5  |
| 74      | Tyr        | 0.93 | 0.75 | 1.40 | 16.4 | 24.9  | 21.5  |
| 76      | Pro        | 0.51 | 0.82 | 0.67 | 8.0  | 3.5   | 7.2   |
| 82      | Phe        | 0.62 | 0.68 | 0.38 | 16.1 | 4.5   | 14.5  |
| 97      | Tyr        | 0.44 | 1.24 | 1.54 | 16.4 | 20.3  | 12.7  |

**Heme group**

| Distances | Plane normals |
|-----------|---------------|
| O/I  | O/R  | I/R  | O/I  | O/R  | I/R  |
| 0.58 | 0.76 | 0.72 | 3.7 | 0.6 | 3.9 |

**Average**

| Distances | Plane normals |
|-----------|---------------|
| O/I  | O/R  | I/R  |
| 0.60 | 0.85 | 1.01 | 11.2 | 13.4 | 14.5 |

**Fig. 19.** Contrasting resolution in histidines 18 and 26. a to c, cross-sections through the ring plane of histidine 18 in the outer, inner, and reduced molecules. d to f, corresponding cross-sections through histidine 26. Histidine 26 lies on the surface of the molecule, with no obvious biological role. Its ring plane orientation is not as well defined as that of histidine 18, which is buried in the molecular interior and which acts as a ligand to the heme iron atom.
where one map contained a neighboring protein molecule and
the other contained either a neighbor in a different orientation
or intermolecular solvent. In contrast, the region within the
interior of the molecule under study was quieter, with small
positive and negative peaks that probably represented noise in
the original electron density maps or slight errors in rotation
parameters for the superposition. Most important, there were
no difference map features that could be said with assurance to
represent genuine conformational differences between the
molecules, aside perhaps from surface groups extending into
the solvent. Examination of these difference maps was the
final argument convincing us that no conformational differ-
ences were visible at the 2.0 A resolution stage.

The lesson from the entire cytochrome c structure analysis
to this point is that the higher the resolution and the greater
the accuracy of the analysis, the fewer are the observed differ-
ences between ferricytochrome and ferrocytochrome mole-
cules. It is possible that continued refinement with 1.5 A data
(now being collected) may ultimately indicate conformational
differences that clearly lie outside the limits of accuracy of this
analysis. But these at most would be only small breathing
motions of the polypeptide chain, or small rotations of side
groups of the order of 15° or less. The old picture of massive
conformational changes between oxidized and reduced mole-
cules (15) was the consequence of wrong interpretation of a
poor low resolution map, and should now be laid to rest. In the
mitochondrial electron transport chain, cytochrome c appears
to be only an electronic component, and not a mechanical
relay.

Acknowledgment — We would like to thank John Chambers
for making his minicomputer programs available to us, and
for discussions about the strategy of refinement.

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