MAPPING AN ELECTRON TRANSFER SITE ON CYTOCHROME *c*

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1. Introduction

Intensive investigation of the cytochrome *c* molecule has failed to produce an accepted description of the electron transfer mechanism. It is assumed that areas on the surface of the molecule are involved in specific interactions with physiological oxidants and reductants. Identification of these areas may help to define the routes of electron exit and entry.

The conventional approach to this problem has been to selectively modify residues and investigate the effect of such modification on the reactivity of the cytochrome *c* with cytochrome oxidase or cytochrome *c* reductase. The purification of molecular species modified at a single position is possible but difficult [1] and conflicting results have been obtained when different reagents have been used to modify a particular residue. Also it is crucial to show that the integrity of the molecule is not affected by a modification.

For these reasons an alternative approach has been used involving the protection of cytochrome *c* by a physiological electron acceptor with which it is known to form a complex and the exposure of this complex to conditions which favour methylation of the available lysine residues. This is therefore analogous to the established method of active site protection by substrates, although in this case relatively large areas of the protein surface may be involved.

2. Materials and methods

Reductive methylation of lysine residues was carried out by the method in [2]. Horse cytochrome *c* (Sigma grade VI, 2 μmol) and yeast cytochrome *c* peroxidase (3 μmol, prepared by the method in [3]) were dissolved in 50 ml 1 mM sodium phosphate, pH 7.0, containing 2 mM NaCl. Sodium borohydride (14 mg) was added and the pH was allowed to rise to 8.3 and then maintained at this value by addition of 0.1 M HCl from a Radiometer autotitrator. Formaldehyde (65 μl) was added in 5 μl portions every 2 min to this solution at 0°C. The methylated cytochrome was adsorbed on CM cellulose, eluted with 10 mM sodium phosphate containing 0.5 M NaCl and the haem group was removed and chymotryptic digestion carried out as described [4]. Peptides were purified by standard electrophoretic methods.

The extent of methylation of lysine residues was determined by amino acid analysis after acid hydrolysis according to the formula

$$\text{Extent of methylation (EM)} = \frac{2 \times [\text{dimethyllysine}] + 1 \times [\text{monomethyllysine}]}{2 \times [\text{lysine}]} \times 100$$

The control experiment contained ovalbumin (sigma grade VI, 3 μmol) instead of the cytochrome *c* peroxidase and from the extent of methylation in the presence and absence of the peroxidase, the % protection was calculated from the formula

$$\%\ \text{protection} = \frac{\text{EM (control)} - \text{EM (cytochrome } c \text{ peroxidase)}}{\text{EM (control)}} \times 100$$

The % protection was calculated in this way for peptides containing a single lysine residue or for figures for all lysines in a peptide protected. For 1

Table 1
Methylation of the lysine residues of cytochrome *c*

Residue	Extent of methylation in the presence of		% Protection of individual residues		
	Cyt. *c* peroxidase	Ovalbumin	1 Residue protected	2 Residues protected	3 Residues protected
(5,7,8)	33	42	+ 64	+32	+21
(13)	40	83	+ 52		
(22,25)	22	24	+ 17	+ 8	
(27)	13	20	+ 35		
(39)	22	26	+ 15		
(53)	10	9	− 11		
(55)	8.2	8	− 3		
(60)	37	33	− 12		
(72,73)	17	27	+ 74	+37	
(79)	5.5	8.7	+ 37		
(86,87,88)	21	39	+139	+69	+46
(99,100)	27	25	− 16	− 8	

Figures are percentages calculated on the basis of the two equations given in the text

out of 2 lysines protected this figure is multiplied by 2; for 1 out of 3 it is multiplied by 3; and for 2 out of 3 it is multiplied by 3/2. These figures are given in table 1.

3. Results

The figures of table 1 show that certain lysine residues are less available for methylation in the presence of cytochrome *c* peroxidase and also that there is considerable variation in the inherent reactivity of lysine residues under these conditions. It is not yet possible to decide which residue or residues are involved when more than one lysine is present in a peptide but the columns of table 1 indicate that if a level of about 50% protection of individual residues has occurred there may be one or two protected residues in the groups (5,7,8) and (72,73) and two or three in the group (86,87,88).

Thus lysine residues which become less available for methylation in the presence of cytochrome *c* peroxidase are the single residues 13 and 27 and members of the groups (5,7,8), (72,73) and (86,87,88). Lysines unaffected by complex formation are the residues 53,55,60 and the pair 99,100. In fact a small decrease in methylation of these lysines was observed in the control experiment and this may be due to increased competition for reagents by the higher concentration of available lysines present.

There are problems in the interpretation of the other results which will be discussed more fully in a subsequent paper. Lysine 79 shows a large percentage decrease in methylation in the presence of the cytochrome *c* peroxidase consistent with its being a protected residue. However, the absolute change in methylation is only 3.5% and it is possible that differential losses of the methylated and unmethylated forms of this peptide during purification may account for this small difference. Similar comments may apply to residues (22,25). Residue 39 is the most serious problem. This residue is apparently protected in the complex although to a lesser extent than others but this is not consistent with its position in the tertiary structure where it is situated at the 'rear' of the molecule (fig.1). The results for table 1 are the results from two single experiments but other experiments gave similar patterns of methylation except with regard to residue 39. In some cases this residue appeared protected while in others it did not. This result can not be explained.

4. Discussion

Cytochrome *c* and yeast cytochrome *c* peroxidase

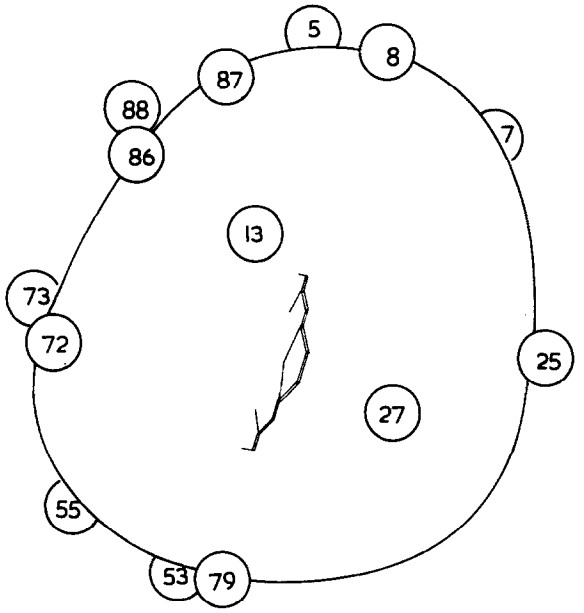

Fig.1. Diagrammatic representation of the 'front' surface of the horse heart ferricytochrome *c* molecule on the basis of the X-ray crystallographic studies [6]. The partly-exposed pyrrole ring II is shown side on. Circles indicate the approximate position of the lysine ε-amino groups.

were chosen as a model electron transfer complex to study because complex formation had been demonstrated [5] and the system may offer the best prospect for crystallographic analysis [5]. Reductive methylation proceeds under gentle conditions and produces little change in surface bulk and no change in surface charge. Because the lysine residues are little affected in properties the peptide map obtained for digestion of a partially methylated protein is identical to that for digestion of unmodified protein. This is an important point for, to assess the extent of modification of an individual residue, it is necessary to purify all the peptides which contain that residue. An incomplete modification which results in a changed mobility of peptides (e.g., acetylation) would give rise to a very complex peptide map.

The decrease in methylation of certain lysine residues of cytochrome *c* in the presence of cytochrome *c* peroxidase is interpreted as a physical protection of these residues in the complex formed by the two proteins. This interpretation (with the exception of the case of lysine 39 noted above) gives a pattern of protection located on the 'front' surface of the cytochrome *c* molecule using the terminology in [6] (fig.1). The binding area appears to involve a large part of that face since lysines 86 and 27 are about 30 Å apart. These findings are consistent with proposals implicating electron transfer at the exposed edge of pyrrole ring II [7–9] and the single residue modification studies which have shown that lysines 13 [10] and 72 [1] are involved in interaction with cytochrome oxidase while lysine 60 is not [1].

Using this approach it should be possible to compare the protection offered by cytochrome oxidase with that provided by NADH cytochrome *c* reductase in order to resolve the problem of whether electron exit and entry take place at the same site. The method should also have wide applications for other macromolecular interactions such as those between hormone and receptor or antigen and antibody.

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