Phosphate Binding by Cytochrome c

SPECIFIC BINDING SITE INVOLVED IN THE FORMATION AND REACTIVITY OF A COMPLEX OF FERRICYTOCHROME c, FERROUS ION, AND PHOSPHATE*

(Received for publication, October 17, 1978, and in revised form, February 12, 1979)

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Electron transfer from ferrous ion to ferricytochrome c is affected by inorganic orthophosphate under conditions when the reductant, the oxidant, and the anionic effector combine to form a stoichiometric complex (Taborsky, G. (1979) J. Biol. Chem. 254, 5246–5251). The hypothesis that a specific phosphate binding site may be involved was tested by exploiting a known side reaction which accompanies the ferrous ion-ferricytochrome c electron exchange. This reaction results in the modification of threonine side chains, of which horse heart cytochrome c has 10. Most of them are in the vicinity of one or more of the even more numerous cationic side chains of the protein. Since the putative phosphate binding site can be expected to be cationic, it was supposed that adjacent to any potential phosphate binding site there would likely be a threonine residue. (This supposition is generally borne out by an inspection of the cytochrome's three-dimensional structure.) Were the reaction of iron, phosphate, and cytochrome to involve such a cationic site, any adjacent threonine might be expected to undergo the modification reaction preferentially. In fact, one residue, threonine 89, was found to suffer modification about 6 to 20 times more extensively than any one of the other nine threonines. The surface region of which this residue is a part includes the cationic side chains of lysines 86, 87, 88, and arginine 91. Singly or in combination, these side chains are proposed to constitute the specific site involved in the formation of the phosphate-modulated oxidation-reduction complex. The site itself is undoubtedly of biological significance in that it is known to be part of the domain which interacts with other respiratory chain components. As a phosphate binding site it may also serve in some mitochondrial regulating event.

Proteins become oxidatively modified when exposed to ferrous ion in the presence of air (1). The reaction was shown to occur with a variety of proteins and has the striking feature that the identity of the reaction product is a function of the ionic composition of the reaction medium. In Tris buffer, the major targets of modification are lysine side chains; in phosphate buffer, the principal effect is on side chains of threonine (and serine) residues.

Compared with other proteins, cytochrome c is a special case. In addition to the effect of its aerobic interaction with ferrous ion on some of its amino acid side chains, it can also accept electrons from the added iron when its heme group is initially in the ferric form. Furthermore, both of these concurrent reactions are facilitated or enhanced when phosphate is present. The facilitation of heme reduction appears to be the consequence of the formation of a stoichiometric complex involving cytochrome c, ferrous ion, and phosphate (2). The enhanced susceptibility of its threonine(s) to oxidative modification, compared with other proteins (1), implied the possibility that this reaction too reflects the specific interaction of the heme protein with ferrous ion and phosphate. A specific interaction being potentially of functional significance, we sought to establish whether any of the numerous threonine residues of cytochrome c show exceptional reactivity. We found this to be the case, and we present the relevant evidence in this paper. The results are interpreted in terms of a specific phosphate binding site on the surface of the cytochrome c molecule.

EXPERIMENTAL PROCEDURES

Ferricytochrome c was reduced with excess ferrous sulfate in medium phosphate buffer, pH 7.5, in air. Under the conditions of these experiments, the conversion of ferricytochrome c to the ferro-form is nearly complete (about 90%), without an opportunity for the reduced heme iron to undergo appreciable autooxidation (1, 3). We had also ascertained earlier that these conditions ensure the essential completion of the oxidative side reaction affecting threonine residues of the protein (1).

After the reduction of the heme iron of cytochrome c and the concurrent oxidation of threonine side chains to their keto derivative were complete, the reaction mixtures were treated with NaB'Hi, in order to generate tritiated threonine wherever the residue had been modified in the preceding reaction. Typically, a large molar excess (relative to the protein) of reducing agent was added and the reaction allowed to proceed under conditions sufficient for the completion of threonine regeneration (1). The radioactive labeling reaction was quenched with acid, and the protein solution was freed of low molecular weight components by gel filtration.

The labeled protein was fragmented specifically, with chymotrypsin or with cyanogen bromide. The fragment mixtures were fractionated by ion exchange chromatography or gel filtration, or both. In some cases, a second fragmentation was carried out. Our purpose was to obtain peptides which contained only a single threonine residue within their structure. Peptides were analyzed for amino acid content. Total radioactivity, and the radioactivity associated specifically with the threonine residues. If amino acid analysis indicated the need for further purification, additional chromatographic steps were used. Peptide purification was carried to the point where the analytical

* This work was supported by a Research Grant (PCM 72-01998) of the National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Selective modification of threonine residues of cytochrome c

Data are based on the specific radioactivity of threonine residues, determined after initial oxidative modification of their side chains, followed by their reduction with NaB\(_{3}\)H\(_4\), fragmentation of the labeled protein, and isolation of the several peptides which contained individual threonine residues. The data for each threonine are standardized relative to the most heavily labeled residue, Thr-89, which was isolated in the same experiment. This permits a direct comparison of all activity data which were actually obtained in three different experiments. Thr-89 was isolated in all of them, once via the chymotryptic cleavage procedure and twice via prior cyanogen bromide cleavage, yielding peptides CT7c and CN2CT1, respectively. Thr-88 and Thr-102 were also isolated in replicate experiments. They provide a measure of the reproducibility of the results. These replicates are all activity data which were actually obtained in three different reactions occurring on account of a presumed general susceptibility of the peptide to threonine modification. One residue, Thr-89, is particularly susceptible to oxidative modification under similar conditions, in proteins other than cytochrome c, not including, of course, Thr-89. The S.D. is indicated.

| Threonine position* | Peptide segment | Relative activity of threonine |
|---------------------|-----------------|------------------------------|
| Position* | Designation | % |
| 10 | 14-22 | CT1T3 | 9.7 |
| 28 | 27-33 | CT8b | 6.0 |
| 40 | 37-46 | CT5e | 4.9 |
| 47 | 47-46 | CT3d | 5.0 |
| 58 | 54-59 | CT6b*2 | 17.3 |
| 63 | 60-64 | CT1b*4 | 11.1 |
| 78 | 66-80 | CN3 | 10.3 |
| 78 | 66-80 | CN3 | 15.4 |
| 78 | 66-80 | CN3 | 15.4 |
| 78 | 66-80 | CN3 | 15.4 |
| 78 | 66-80 | CN3 | 15.4 |
| 78 | 66-80 | CN3 | 15.4 |
| 78 | 66-80 | CN3 | 15.4 |
| 78 | 66-80 | CN3 | 15.4 |
| 78 | 66-80 | CN3 | 15.4 |
| 78 | 66-80 | CN3 | 15.4 |
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| 78 | 66-80 | CN3 | 15.4 |
| 78 | 66-80 | CN3 | 15.4 |
| 78 | 66-80 | CN3 | 15.4 |

Average of all threonines except Thr-89: 15.2 ± 5.6

DISCUSSION

The preferential modification of one threonine residue of cytochrome c, under the conditions described here, suggests a mechanism which involves the interaction of iron and phosphate with a particular surface site on the cytochrome. This view is reinforced by our previously described finding (1) that the reduction of the heme iron by ferrous ion is facilitated by phosphate because cytochrome c, phosphate, and iron constitute a complex within which electron transfer occurs between the added iron and the heme. Phosphate being the apparently specific promoter of both reactions, it is reasonable to suppose that its effect is a reflection of a specific phosphate binding ability of the cytochrome. Presumably, the interaction involves a particular, positively charged, anion-binding locus on the protein surface.

Cationic clusters, formed by lysine and other positively charged amino acid side chains on the cytochrome c surface, have often been ascribed significance regarding the native structure of the protein, its interactions, and its physiological function. An anion binding site is associated with the crystalline protein (4, 5). In solution, P, and nucleotides appear to bind at two loci (6). NMR data implicate His 26 in this binding site, an extent appreciably below the extent of modification of residues in the cytochrome c. The specific susceptibility of Thr-89 appears to account for this difference.
Phosphate Binding Site of Cytochrome c

A site near this histidine, possibly involving the neighboring Lys-27, and an additional site centered on Lys-87, emerged as likely Pi binding sites from experiments concerned with electrostatic interactions of cytochrome derivatives with selectively modified lysine side chains (8).

Nucleotide binding is of particular interest to us because we found earlier that ATP and ADP, but not AMP, affect cytochrome reduction by Fe²⁺ in a manner similar to P₃ (3). Cytochrome c affects the phosphorus resonances of ATP and ADP, but not AMP (9). A similar, differential response to nucleotides is revealed in terms of their enhancement of the 695 nm absorption of the form of ferricytochrome c which is dominant at neutral pH (10). A reducing nucleotide, NADH, also prefers that form (11).

Cationic sites are implicated in the promotion of electron exchange with anionic reactants. Iron hexacyanide, in stable complex with cytochrome c, shows enhanced electron exchange (12) and binds at sites which depend on free lysine side chains (13). Speculations concerned with the involvement of Lys-79 have come into question recently (14, 15), but alternatives have been proposed in terms of two clusters formed by Lys-86 and -88, Arg-91, and by Lys-5, -7, -8, and -7 or -13 (16). One of these may be identical with one of the P₃ sites. This would be consistent with the reported competition between P₃ and hexacyanide (16). It also competes with dithionite (17). This site may be identical with one of the hexacyanide sites; kinetic differences between the two anionic reductants have been attributed to differences in the lifetimes of their respective complexes with the cytochrome (18).

Such complex formation is, of course, central to our interpretation of the Fe²⁺-cytochrome interaction. The formation of an analogous oxidation-reduction complex would be consistent with the kinetics of the reduction of cytochrome c by catecholate (19). A similar complex may be involved in the reaction of cytochrome c with cortisol (this reaction being formed by Lys-86 and -88, Arg-91, and by Lys-5, -8, -87, and -7 or -13 (16). One of these may be identical with one of the P₃ sites. This would be consistent with the reported competition between P₃ and hexacyanide (16). It also competes with dithionite (17). This site may be identical with one of the hexacyanide sites; kinetic differences between the two anionic reductants have been attributed to differences in the lifetimes of their respective complexes with the cytochrome (18).

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Anionic free radical reductants have also been considered to bind at positively charged loci (21), but a unique interpretation of their reactions is elusive because they may proceed by a variety of mechanisms, at diverse sites and with various cytochrome conformers (22-24).

Anions affect cytochrome reduction when the reductant is a positive ion, the reaction under study here being an example of type catalysis of cytochrome reduction by Cr³⁺ is accounted for in terms of anionic bridges linking the reductant either directly to the heme iron or to another cytochrome site (such as the heme edge), depending on the relative rates of heme iron ligand replacement and the reduction (25). P₃ binding to a chromium-cytochrome complex has been shown, but not the site of this interaction (26). (In the absence of an interacting anion, Cr has been shown to be bound to Tyr-67 and Asn-52 (27).)

Anionic metal complexes will not bind to cytochrome c indiscriminately. We surmised earlier that certain coordination complexes of Fe³⁺ (e.g. EDTA) may be unsuited for complex formation with the cytochrome (3). It is now known that blockage of all lysine side chains of cytochrome c is without consequence on its reduction by Fe(EDTA)³⁻ (28), when compared with the reduction rate measured with the unmodified protein (29).

Functional involvement of cationic sites is well established for interactions of cytochrome c with components of the electron transfer chain. Findings such as the P₃-dependent dissociation of cytochrome c from Keilin-Porter preparations (30), the effects of P₃ on the rate of cytochrome oxidation by cytochrome oxidase (31), and the effects of polyvalent cations (30, 32), pH, buffers, and ionic strength (33) on the reductase and oxidase reactions, left little doubt early on that electrostatic interactions are essential. More recently, it has become clear that these interactions involve specific loci of positive charge.

The domain of the cytochrome c surface which interacts with cytochrome oxidase is now defined with appreciable precision. It involves lysine residues 8 (34, 35), 13 (34-38), 25 (39), 27 (34, 35), 79 (34), and 87 (35). In contrast, residues 22 (35, 37, 39), 39 (35), 55 (38), 60 (35), 99 (35, 38), and 100 (34) appear to fall outside the interaction domain. (Lysine residues 5, 7, 53, 73, 86, and 88 have not been covered by these efforts.) It is noteworthy that four of the five invariant lysine residues and a conservatively variable one (40) are implicated in the interaction.

This domain includes, at its periphery, the P₃ binding site at Lys-87 suggested by an earlier study (8). That this may be a physiologically significant site is suggested by the observation that certain anions, P₃, and nucleotides among them, affect the kinetics of the cytochrome c-cytochrome oxidase reaction at concentrations within the physiological range (41). The site may be of consequence in terms of regulation.

The outlines of the interacting domain with respect to cytochrome reductase are also emerging. Extensive blocking of lysine side chains prevents cytochrome c from complexing with cytochrome c₁ (42). In this interaction, the specific involvement of lysines 8, 13, 27, 72, and 79 has been indicated (43), whereas lysines 22, 25, 55, 99, and 100 appear not to be involved (39, 43). It is striking to what extent the two domains of the oxidase and reductase, respectively, appear to overlap. This is of obvious significance for the elucidation of the biological mechanism of electron transfer involving cytochrome c (for relevant discussions, see Refs. 35, 43-45).

In most cases of observed anion binding to cytochrome c, the precise location of the binding site is either a matter of speculation or is inferred from findings of interference with binding by the chemical modification of potential sites. The cytochrome c-Fe³⁺-P₃ system appeared to provide an opportunity for the identification of a phosphate binding site in a cytochrome molecule of which the functional integrity remains unimpaired. In fact, the cytochrome c-phosphate complex has an enhanced electron transfer capability. We consider that the modified residue, Thr-89, marks the site of phosphate binding. This is based on the supposition that any side chain which would be preferentially modified when the protein interacts with Fe³⁺ and P₃ must be in the vicinity of the site of interaction.

The results presented in this paper are well accommodated within the overall context of our knowledge regarding cationic binding sites on the cytochrome surface. Cationic charges proximal to Thr-89 are provided by lysines 86, 87, 88, and arginine 91. We propose that the putative phosphate binding site involved in the reactions described here and earlier (2) is constituted of these residues in some combination. It is noteworthy that, of these cationic side chains, two (Lys-87 and Arg-91) are invariant in the evolutionary sense (40). It is noteworthy too that a recent study of arginine models has shown them to be particularly suitable for the specific binding of phosphate (49). This proposal is in specific agreement with one of the two likely phosphate binding sites which emerged from experiments concerned with the electrostatic interactions of cytochrome c derivatives with selectively modified side chains (8).

If, as we believe, this phosphate binding site is involved in the binding of Fe³⁺ and the subsequent electron transfer to the heme iron (2), then the location of the binding site would seem to require a mechanism of electron transfer which is indirect. The binding site is too distant to accommodate direct reductant-oxidant contact between Fe³⁺ on the one hand and...
It could be argued that, even if the threonine oxidation is a consequence of the aerobic interaction of cytochrome c with Fe$^{3+}$ and P, at a specific site, the site of this interaction (which is also taken to be the site of formation of the actual oxidant) may be removed from the location of the modifiable threonine—the oxidant might migrate. Our results cannot definitively rule out this possibility, although there is cause to consider it most unlikely. The mechanism of the oxidation is not known, but it seems clear, in view of its dependence on O$_2$ that it is of the nature of a ligand autoxidation. Such reactions are well documented (as referred to in Ref. 1 and other references cited therein; a more recent example of the facile autoxidative dehydrogenation of an iron ligand is provided in Ref. 46). But a ligand autoxidation would make the direct availability of the oxidizable component a necessity at the site of the formation of the oxidant. Also, it seems that if the oxidant were to migrate, the preference for Thr-89 reflects only a fortuitous juxtaposition of that particular residue to a phosphate binding site which may lack necessity at the site of the formation of the oxidant (40, 47, 48; an exception is Thr-49, to which we referred earlier as an "internal" residue).

It might also be argued that the preferential modification of Thr-89 reflects only a fortuitous juxtaposition of that particular residue to a phosphate binding site which may lack functional significance; "significant" binding may be elsewhere, remote from the Thr-89 locus and remote also from other threonines, which would be, therefore, nonreactive in this system. However, the 10 threonine residues of cytochrome c find themselves in nearly every case in the vicinity of one or more of the even more numerous positively charged side chain residues (19 lysines, 3 histidines, and 2 arginines (47)), as revealed by the x-ray crystallographically determined structure of the protein (40, 48). In addition, the finding that phosphate also promotes the electron transfer between Fe$^{3+}$ and the heme group within a cytochrome c-Fe$^{3+}$-P complex (2) reinforces the view that the site marked by modified Thr-89 is a functionally significant phosphate binding site.

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Phosphate Binding Site of Cytochrome c

Supplemental Material

to

Phosphate Binding of Cytochrome c

SPECIFIC BINDING OF PHOSPHATE ION TO CYTOCHROME C: THE RELATIONSHIP BETWEEN RADIOACTIVITY OF A COMPLEX OF PHOSPHORUS-C15, TETRAMIN, AND CYTOCHROME C

George Houghton and Kenneth Mcleod

EXPERIMENTAL PROCEDURES

Reduction of ferricytochrome c by Fe++ and subsequent modification of the protein by dialysis against phosphate buffer. The preparation was described in Ref. 1.

Reaction mixture consisted of 0.08-0.25 M ferricytochrome c (Sigma, Type III) and a 1% solution of 2-mercaptoethanol. Buffer: 0.05 M phosphate, pH 7.4. The mixture was stirred at room temperature.

Reduction of modified cyanochrome c complex to pH 7.4. The protein mixture was incubated at 30°C for 15 min, and the redox state was determined by ferrous ion reduction.

Reaction mixture contained 10-20 ml ferricytochrome c (Sigma, Type III) and a 1% solution of 2-mercaptoethanol. Buffer: 0.05 M phosphate, pH 7.4. The mixture was stirred at room temperature for 20 min and the reaction was then stopped by addition of N,N-Dimethylformamide (DMF). Dimerization of unreacted cytochrome c was prevented by the addition of 10% (v/v) ethanol. The reaction mixture was then centrifuged and the resulting supernatant was used for the reaction mixture.

Fragmentation of cytochrome c by cyanogen bromide. The cytochrome c solution was added to a solution of methanol and water (1:1) and the mixture was then treated with cyanogen bromide (Cyanogen, 100 ml). The reaction mixture was then centrifuged, and the supernatant was used for the fragmentation mixture.

Fragmentation of cytochrome c by cyanogen bromide. Cyanogen bromide (100 ml) was added to the cytochrome c solution. The reaction mixture was then centrifuged, and the supernatant was used for the fragmentation mixture.

Infrared absorption of cytochrome c at 1690 cm⁻¹. The absorbance of the cytochrome c solution was determined at 1690 cm⁻¹ using a Cary Model 60 spectrophotometer.

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Infrared absorption of cytochrome c at 1690 cm⁻¹. The absorbance of the cytochrome c solution was determined at 1690 cm⁻¹ using a Cary Model 60 spectrophotometer.
**FIG. 2.** Gel filtration of pooled, ion-exchange chromatographically separated fractions derived from a tryptic digest of cytochrome c. CT-fractions which yielded tryptic peptides without further purification (fractions 0-40) were designated as CT-fractions, (fractions 0-40) were designated as CT-fractions; the other fractions (CTb-d) were fractionated as indicated in the Experimental Procedures. A representative profile is shown in Fig. 3. The latter fractions were designated C1 and C2, respectively. The latter fractions were designated C1 and C2, respectively.

**FIG. 3.** Gel filtration of pooled, ion-exchange chromatographically separated fractions derived from a tryptic digest of cytochrome c. Peptides derived from tryptic digestion products. The tryptic digestion products were separated on a Bio-Gel column C1a and C2a. The latter fractions were designated as C1b, C1c, C1d, and C1e, respectively. For further details, see the legend for Fig. 2.

**FIG. 4.** Gel filtration of pooled, ion-exchange chromatographically separated fractions derived from a tryptic digest of cytochrome c. Peptides derived from tryptic digestion products. The tryptic digestion products were separated on a Bio-Gel column C1a and C2a. The latter fractions were designated as C1b, C1c, C1d, and C1e, respectively. For further details, see the legend for Fig. 2.

**FIG. 5.** Gel filtration of pooled, ion-exchange chromatographically separated fractions derived from a tryptic digest of cytochrome c. Peptides derived from tryptic digestion products. The tryptic digestion products were separated on a Bio-Gel column C1a and C2a. The latter fractions were designated as C1b, C1c, C1d, and C1e, respectively. For further details, see the legend for Fig. 2.
Phosphate Binding Site of Cytochrome c

TABLE II

Distribution of Radiactivity Among Peptide Fragments of Cytochrome c After Initial Separation

| Peptide Designation | Number of Fragments | Radioactivity Activity Value |
|---------------------|---------------------|-----------------------------|
| CIT                 | 24                  | 3.0                          |
| CIT-1               | 3                   | 8.5                          |
| CIT-2               | 3                   | 1.1                          |
| CIT-3               | 3                   | 1.4                          |
| CIT-4               | 3                   | 2.2                          |
| CIT-5               | 3                   | 2.6                          |
| CIT-6               | 3                   | 3.2                          |
| CIT-7               | 3                   | 3.4                          |
| CIT-8               | 3                   | 3.8                          |
| Total               | 24                  | 11.6                         |

Note: The very low total radioactivity of this fraction, it was checked without further analysis.

3. This is a result of a separate analysis of the unfiltered samples. The relatively low recovery of activity in the eluted supernatants is quite obvious. As noted earlier (11), about 30% of the total radioactivity of the intact protein is lost by exchange with sodium acylphosphates. The distribution of the remaining 70% of the radioactivity in various hydrolysates was not determined. The form of the radioactivity profile of the unfiltered samples obeyed the same chromatography of the original sample. 4. The authors believe that the fractions could be eluted with strong alkali in the absence of the presence of potassium acetate and only then recovered the substantial percentage of activity. It is quite possible that this fraction did not contain dephosphorylated proteins. For this reason, this fraction was rejected for further investigation. The possibility of artifacts arising from the use of hydrolysates was considered, as we noted earlier (11).
Phosphate binding by cytochrome c. Specific binding site involved in the formation and reactivity of a complex of ferricytochrome c, ferrous ion, and phosphate.

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J. Biol. Chem. 1979, 254:7069-7075.