Enzymic Activities of Covalent 1:1 Complexes of Cytochrome *c* and Cytochrome *c* Peroxidase†

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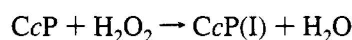
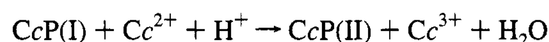
ABSTRACT: We have obtained several cysteine mutants in or around the cytochrome *c* peroxidase binding domain of rat and yeast iso-1 cytochrome *c* by site-directed mutagenesis. These cysteine residues were specifically labeled with the bifunctional photoactive cross-linker 4-azidophenacyl bromide (APB). 1:1 covalent complexes of cytochrome *c* peroxidase and cytochrome *c* were generated by cross-linking these specifically labeled cytochromes *c* to cytochrome *c* peroxidase, and the 1:1 complexes were purified. Steady-state kinetic studies of the purified 1:1 complexes with free yeast and horse cytochromes *c* showed the following: (1) Cytochrome *c* peroxidase has two distinct catalytic sites—a high-affinity and a low-affinity site. (2) Other than the difference in affinity, the binding of substrate at the low-affinity site is similar to that at the high-affinity site, with yeast cytochrome *c* interacting more strongly than the horse protein, the binding of both substrates being sensitive to ionic strength, and both sites able to transfer electrons. (3) HPLC chromatography of purified 1:1 complex showed multiple forms of 1:1 complexes, supporting the idea of multiple possible interactions between cytochrome *c* and the high-affinity site on cytochrome *c* peroxidase. (4) An allosteric or electrostatic effect exists between the two substrate binding sites, the binding of cytochrome *c* to the high-affinity site decreasing the binding affinity of the low-affinity site to cytochrome *c*. The higher the equilibrium binding affinity of the mutant cytochrome *c* to the peroxidase, the larger the apparent allosteric/electrostatic effect when that mutant protein is covalently bound to the high-affinity site of the enzyme. Furthermore, different locations of the covalently bound cytochrome *c* at the high-affinity site on the enzyme surface result in different degrees of allosteric/electrostatic effect. The presence of two active sites on the enzyme allows a simple interpretation of some of the differences in the steady-state kinetic behavior of cytochrome *c* peroxidase with horse and yeast iso-1 cytochrome *c*.

Yeast cytochrome *c* peroxidase (C*c*P)[1] catalyzes the peroxide-dependent oxidation of ferrocytochrome *c* (Cc^{2+}) as illustrated in the following multistep reactions (Coulson et al., 1971):



$$CcP + H_2O_2 \rightarrow CcP(I) + H_2O$$



$$CcP(I) + Cc^{2+} + H^+ \rightarrow CcP(II) + Cc^{3+} + H_2O$$

$$CcP(II) + Cc^{2+} + H^+ \rightarrow CcP + Cc^{3+}$$

First, the native Fe^{3+}–heme C*c*P undergoes a 2-equivalent oxidation by hydrogen peroxide to form an oxidized intermediate called compound I [C*c*P(I)] (Jordi & Erman, 1974) which consists of a ferryl (Fe^{4+}) iron (Hagar et al., 1972) and an amino acid free radical (Yonetani et al., 1966; Bosshard et al., 1991) associated with Trp 191 (Sivaraja et al., 1989; Erman et al., 1989). In the next step, a molecule of Cc^{2+} reduces compound I by one electron to form compound II [C*c*P(II)]. Then a second molecule of Cc^{2+} reduces C*c*P(II) by one electron to form the native enzyme. Over the last 2 decades, numerous studies have addressed the mechanism of electron transfer in the C*c*–C*c*P system; these have been very well covered in a recent paper [see Stemp and Hoffman (1993) and references cited therein] to which the reader is referred.

The binding site on C*c* for C*c*P has been well studied through chemical modification (Kang et al., 1978) and site-directed mutagenesis (Hazzard et al., 1988a). It is located on the upper left of the front surface of the protein and includes a ring of positively charged lysine residues surrounding the exposed heme edge which abuts the surface of the molecule and is available to solvent (Kang et al., 1978). The binding site on C*c*P for C*c* has also been examined. Poulos and Kraut (1980) modeled a 1:1 complex based on the crystal structures of C*c*P and tuna heart C*c* by optimizing interactions between complementary charged groups of the two proteins with a computer-graphics system. In the model, the binding site on C*c*P contains a ring of negatively charged aspartate residues with a spatial distribution that is complementary to the distribution of the highly conserved lysines in the binding site on C*c*. However, Pelletier and Kraut

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[1] Abbreviations: C*c*P, cytochrome *c* peroxidase; C*c*P(I), compound I of C*c*P; C*c*P(II), compound II of C*c*P; C*c*, cytochrome *c*; Cc^{2+}, ferrocytochrome *c*; Cc^{3+}, ferricytochrome *c*; RN*c*, rat C*c*; SC*c*, yeast iso-1 C*c*; APB, 4-azidophenacyl bromide; YEp*RC9* and YEp*CYC1*, YEp13 plasmid carrying *RC9* and *CYC1*, the rat and yeast iso-1 C*c* gene, respectively; K8C, K25C, K87C, V28C, D50C, and G83C, mutations at Lys 8, Lys 25, Lys 87, Val 28, Asp 50, and Gly 83 in which these residues are replaced by Cys; C102S, mutation in which Cys 102 is replaced by Ser.

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(1992), who recently solved the structures of 1:1 cocrystals of CcP with either SCc or horse Cc, observed slightly different binding sites for the two proteins that also differed from but overlapped with the putative Poulos–Kraut site. The crystallographic binding site was predicted to contain an efficient electron transfer pathway (Beratan et al., 1992).

One of the controversial issues has been the stoichiometry of complex formation. Each CcP(I) reduction cycle involves two molecules of Cc^{2+} as well as two separate redox centers on the enzyme, suggesting that CcP might have two sites for binding to and electron transfer from Cc^{2+}, as originally proposed by Kang et al. (1977). On the other hand, if CcP reacts with Cc at only one site, the postulated kinetic mechanisms nmecessarily become complicated (Kim et al., 1990). Nevertheless, the concept of a Cc:CcP functional binding stoichiometry of 1, a ratio first observed by ultracentrifugation and molecular sieve chromatography by Mochan and Nicholls (1971), was supported by numerous investigations over the following 2 decades. These included binding (Mochan & Nicholls, 1971; Gupta & Yonetani, 1973; Leonard & Yonetani, 1974; Erman & Vitello, 1980; Vitello & Erman, 1987; Corin et al., 1991), NMR (Satterlee et al., 1987; Moench et al., 1987, 1992), chemical modification (Waldmeyer et al., 1982; Bechtold & Bosshard, 1985), kinetic (Kim et al., 1990; Corin et al., 1991), and cross-linking (Waldmeyer et al., 1982; Waldmeyer & Bosshard, 1985; Moench et al., 1993) studies. However, the existence of a second site for Cc on CcP could not be ruled out. Indeed, at relatively low ionic strengths with horse Cc and higher ionic strengths with SCc, the steady-state kinetics of the reaction with CcP are biphasic, and the apparent K_m values for the high- and low-affinity phases correspond rather closely to the K_d values measured by gel filtration for the binding of the first and second molecule of Cc to CcP, respectively (Kang et al., 1977). Similarly, Kornblatt and English (1986) showed that prophyrin Cc, like Cc, yields a 2:1, as well as a 1:1, complex with CcP. Furthermore, Brownian dynamics computer simulations of the association of CcP with Cc (Northrup et al., 1988) show several potential energy minima in the region of the enzyme postulated (Poulos & Kraut, 1980) or observed (Pelletier & Kraut, 1992) to interact with Cc in the 1:1 complex. This would indicate that in the high-affinity domain, Cc can occupy a number of apparently mutually exclusive areas. A separate potential energy minimum, centered around residues 148 and 150, both aspartic acids, may represent the location of the low-affinity site. Electrostatic calculations (Northrup & Thomasson, 1992) show that the interaction at the high-affinity site is more stable than at the low-affinity site.

Most recently, Stemp and Hoffman (1993), measuring fluorescence quenching of triplet state ZnCcP by Cc, demonstrated that there are two binding domains on the enzyme: the high-affinity domain exhibiting strong quenching without significant electron transfer and the low-affinity domain quenching mainly by electron transfer. These conclusions were confirmed by following photoinduced electron transfer between CcP and ZnCc (Zhou & Hoffman, 1993), again demonstrating two binding and electron transfer sites for the substrate, Cc, on the enzyme, with the binding affinities varying by about 10^3.

Chemical cross-linking data have also been used to address the issue of binding stoichiometry. Bisson and Capaldi (1981) found that the 1:1 covalent complex formed between arylazidolysine 13 horse Cc and CcP has no electron transfer activity toward horse Cc. Waldmeyer and Bosshard (1985) cross-linked CcP and horse Cc with a water-soluble carbodiimide and found that the covalent 1:1 complex had only 5% activity left toward horse Cc. However, the loss of activity appears to have been due to modification of enzymically important carboxyl groups on CcP in the cross-linking reaction (Moench et al., 1987). A more recent cross-linking study (Moench et al., 1993) also suggested that the stoichiometry is 1:1. In all these cross-linking studies, the 1:1 covalent complex is the major species after cross-linking, but 2:1 complexes are also observed in the presence of excess Cc, and since the 1:1 complex is enzymically inactive under the conditions and substrate Cc employed, the 2:1 complex is interpreted to be the result of nonspecific cross-linking.

Here, we report the use of mutants of SCc, with a serine instead of the natural cysteine at 102, and cysteine at residue 28, 83, or 87, and of RNc with cysteine at residue 8, 25, 50, or 87, to determine the binding stoichiometry of CcP with Cc and the enzymic activities of their covalent complexes. Our results show that multiple 1:1 Cc–CcP covalent complexes are formed, with the high-affinity site of CcP blocked by the covalently bound Cc, each one of which has a significant but different level of activity with SCc as substrate. The binding of the substrate Cc to the 1:1 covalent complex is affected by the same variables that influence its binding to the high-affinity site of the enzyme, mainly the nature of the Cc, fungal or mammalian, and the ionic strength. Remarkably, the apparent binding affinity of the 1:1 covalent complex for a second molecule of Cc decreases as the original equilibrium binding affinity of the Cc employed in making the 1:1 covalent complex increases, and vice versa. This effect of one molecule of substrate bound at the high-affinity domain on the binding of a second molecule at the low-affinity interaction site is also influenced by the location occupied by the first molecule on the enzyme surface. These phenomena indicate that there is an allosteric or electrostatic effect operating between the high- and low-affinity enzymic sites on CcP.

MATERIALS AND METHODS

Enzymes (including DNA restriction enzymes, kinase, ligase, and polymerase) were purchased from Promega, Boehringer Mannheim, or Stratagene and were used according to manufacturers' instructions. The cross-linker 4-azidophenacyl bromide (APB) was purchased from two sources, Fluka and Sigma, and they were equally effective. Centricons/Centripreps were purchased from Amicon. CcP crystals, prepared as described by Stemp and Hoffman (1993), were a kind gift from Eric D. A. Stemp, Northwestern University.

Site-Directed Mutagenesis. Plasmids YEp*RC9* and YEp*CYC1* are described elsewhere (Koshy et al., 1992). Bacterial strains RZ1032 (*dut, ung*), JM109, and DH5α were used to manipulate DNA. Yeast strain GM-3C-2 (α, *leu2-3, leu2-112, trp1-1, his4-519, cyc1-1, cyp3-1*), which has no functional endogenous Cc genes, was used to express the recombinant Cc genes. Two methods were employed to make Cc mutants. One is the conventional *U*-DNA mutagenesis protocol, as described by Geisselsoder et al. (1987). The other is a PCR mutagenesis method which was developed to simplify the mutagenic procedure. Its principle is

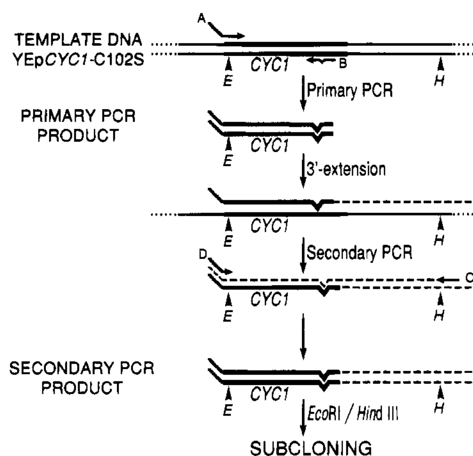

FIGURE 1: Illustration of the PCR mutagenesis procedure. The primers employed are represented by A, B, C, and D. *CYC*1 represents the yeast iso-1 *Cc* gene (thick line), and *E* the *Eco*RI and *H* the *Hin*dIII restriction sites. The thin lines represent sequences flanking the *CYC*1 gene, the dashed lines sequences newly synthesized by PCR or 3′-extension, and the dotted lines segments of plasmid DNA sequences. The dented point (v) indicates the introduced mutation. The procedure is described under Materials and Methods.

illustrated in Figure 1. YEp*CYC1*-C102S (the plasmid that contains *CYC1*, the yeast iso-1 *Cc* gene, in which cysteine 102 had been replaced by serine) was used as template for PCR. Four primers, A, B, C, and D, were employed. Three of them (A, C, and D) were "universal " primers which can be used to make any mutations on this gene. The fourth primer (mutagenic primer B) was specific to each mutation. Primer A was a 40-mer with its 3′-half complementary to a region that contains an *Eco*RI site at the beginning of the *CYC1* coding region and its 5′-half not complementary to any sequences in the template. Primer B was a 25-mer which contains the desired mutation. Primer C was a 23-mer which complements a region that contains a *Hin*dIII site downstream of the *CYC1* coding region. Primer D was a 20-mer that has the same sequence as the 5′-half of primer A. PCR reactions were carried out as described by Maniatis et al. (1989) with *Pfu* DNA polymerase to minimize nucleotide misincorporation. The primary PCR was done with YEp-*CYC1*-C102S as template and the primer pair A and B, generating a PCR fragment that contains part of the *CYC1* coding region and the desired mutation. This fragment was purified electrophoretically on a low-melting agarose gel, a small aliquot was mixed with an equal amount of the original template to perform 3′-extension, and then primer pair C and D were added to perform secondary PCR. In this fashion, only the mutated template will be amplified exponentially. The secondary PCR product was also purified on a low-melting agarose gel, digested with *Eco*RI−*Hin*dIII, and subcloned back into the yeast expression vector YEp*CYC1* for DNA sequencing and protein expression. A similar approach has been used by Merino et al. (1992) to obtain mutants of pUC/M13 vectors.

The construction of yeast expression vectors YEp*RC9* and YEp*CYC1*, transformation of the yeast strain GM-3C-2, large-scale fermentation of the transformed yeast, and purification of the mutant *Cc* proteins were carried out as described by Koshy et al. (1992). HPLC fraction II, the N-terminal nonacetylated *Cc*, was used exclusively.

Labeling and Cross-Linking Reactions. APB, a small, bifunctional, photoactive reagent, was employed as cross-linker. The α-bromoketone moiety reacts specifically with cysteine at neutral pH, and the nitrene that derives from the azide moiety upon exposure to long-wavelength UV (300−350 nm) reacts with various groups that are spatially close (Hixson & Hixson, 1975). The procedure for labeling *Cc* was as follows: mutant *Cc* (2 μmol of HPLC fraction II) containing a free cysteine was fully reduced with an excess of β-mercaptoethanol, passed through a Sephadex G-10 column (equilibrated in 25 mM potassium phosphate buffer, pH 7.0) under argon to remove excess reducing agent, mixed with an excess (more than 10-fold) of APB in the presence of 15% methanol in the same buffer deoxygenated with argon, and kept in complete darkness at room temperature for 90 min. The *Cc* was then collected on a 1 mL CM-cellulose (microgranular, Whatman) column, washed with 10 mM potassium phosphate, pH 7.0, buffer to remove extra APB, and eluted with the same buffer containing 400 mM KCl. The eluent was desalted on a Sephadex G-10 column equilibrated in 10 mM Tris−chloride, pH 6.0. The molar ratio of APB to *Cc* in the product was measured photometrically on a Hitachi 557 recording spectrophotometer. Assuming that the covalent binding of APB to the free cysteine residue on the mutant *Cc* does not change their spectra significantly, the absorption of the compound *Cc*−APB at a given wavelength should be the sum of the absorption of *Cc* and APB at that wavelength. Thus, the molar ratio of [APB]/[*Cc*] can be calculated from the following:

$$\frac{[\mathrm{APB}]}{[Cc]} = \frac{[A_{300} - (P_r\epsilon^{2+}_{300c} + P_o\epsilon^{3+}_{300c})(A^{2+}_{550}/\epsilon^{2+}_{550c})]\epsilon^{2+}_{550c}}{A^{2+}_{550}\epsilon^{\mathrm{APB}}_{300}}$$

$$P_r = \frac{\epsilon^{2+}_{550c}(A_{550}/A^{2+}_{550}) - \epsilon^{3+}_{550c}}{\epsilon^{2+}_{550c} - \epsilon^{3+}_{550c}}$$

$$P_o = 1 - P_r$$

In these expressions, ϵ^{2+}_{550c} and ϵ^{3+}_{550c} are the millimolar extinction coefficients of Cc^{2+} and Cc^{3+} at 550 nm; ϵ^{2+}_{300c} and ϵ^{3+}_{300c} are the millimolar extinction coefficients of Cc^{2+} and Cc^{3+} at 300 nm; $\epsilon^{\mathrm{APB}}_{300}$ is the millimolar extinction coefficient of APB at 300 nm; P_r and P_o are the percentages of Cc^{2+} and Cc^{3+} after the labeling reactions; A_{300} and A_{550} are the absorpions of the labeled *Cc* solution at 300 and 550 nm, while A^{2+}_{550} is its absorption after complete reduction with solid sodium dithionite. APB-modified *Cc* was also analyzed by both reversed-phase (Waters μBONDAPAK C_{18} column) and cation-exchange (Waters PROTEIN-PAK SP5PW column) HPLC to ascertain that the APB molecule was covalently attached to *Cc* at the −SH of the available cysteine.

The 1:1 *Cc*−CcP covalent complex was obtained by mixing CcP with *Cc*−APB in 10 mM Tris−chloride, pH 6.0, buffer at a concentration of *Cc* of about 2−5 μM and a molar ratio for [CcP]/[*Cc*] of about 1.1. The mixture (50−100 mL) was incubated for 5 min and then irradiated for 30 min with long-wavelength UV light from a BLAK-RAY XX15C UV lamp (ULTRA-VIOLET PRODUCTS INC.) at a distance of 1 cm in a petri dish (90 mm diameter). These above procedures were carried out at 0−4 °C in complete darkness.

Purification and Steady-State Kinetics of 1:1 Covalent Complex. After UV exposure, the ionic strength of the cross-linking solution was raised to 80 mM with KCl, and the 1:1 Cc−CcP covalent complex was separated from free CcP, Cc, and 2:1 Cc−CcP complex. CcP was removed on a DEAE-Sepharose (fast flow) column, and Cc and its polymers as well as the 2:1 covalent complex were adsorbed on a CM-Sepharose (fast flow) column. The flow-through containing 1:1 covalent complex was concentrated on a Centriprep-10 ultrafiltration device. The Cc:CcP molar ratio in the purified 1:1 covalent complex sample was determined spectrophotometrically (Mochan & Nicholls, 1971). The products of cross-linking and purification at various stages were analyzed directly by SDS, isoelectric focusing, or electrophoretic titration curve gel electrophoresis using the PhastGel system (Pharmacia), and by cation-exchange HPLC with a Waters 510 system. Chromatography of the 1:1 covalent complex was performed on a CM-silica column (SynChropak CM300, 25.0 × 0.46 cm) at a flow rate of 1 mL/min under a gradient from 10 mM potassium phosphate, pH 5.5, to the same buffer containing 400 mM KCl.

The steady-state enzymic kinetics of CcP and various Cc−CcP 1:1 covalent complexes, using different Cc as substrate, were determined as described by Kang et al. (1977). Fully reduced Cc were used as substrate over concentration ranges of 0.5−50 μM, covering both the high- and the low-affinity phases.

RESULTS

Preparation and Characterization of Cysteine Mutants of Cc. To obtain homogeneous singly-substituted preparations of chemically modified Cc, often employed to study the interactions between CcP and Cc, requires prolonged chromatographies [see, for example, Brautigan et al. (1978)]. When the product of the modification reaction is unstable, as in the present case, this approach is impractical. We therefore chose to replace appropriate surface residues with cysteine through site-directed mutagenesis and then modify the single highly reactive sulfhydryl group for a quantitative yield of a homogeneous product. With the rat protein which carries no free sulhydryl group, four cysteine mutants were made by the conventional *U*-DNA mutagenesis method: RNc-K8C, RNc-K25C, Rnc-D50C, and RNc-K87C. The RNc-K87C mutation is located at the upper left front surface of Cc, well within the binding domain for CcP (Kang et al., 1978). The RNc-K8C and RNc-K25C mutations are located at the upper right and the lower right regions of the front surface, respectively, near the boundary of the binding domain. The RNc-D50C mutation is located at the bottom surface of Cc, outside the binding domain. Three SCc cysteine mutants have been obtained by the PCR mutagenesis method. They are SCc-K87C, SCc-G83C, and SCc-V28C, all located within the binding domain. In these mutants, the single cysteine with a free sulfhydryl in the original SCc, that at position 102, had been replaced by a serine, leaving the new introduced cysteine as the only one available for chemical modification. All the mutant proteins obtained so far have normal UV−visible spectra which suggests that their conformations are not significantly altered. The mutations were confirmed by DNA sequencing. Protein disulfide dimers formed upon oxidation with ferricyanide and were readily monomerized by reduction with β-mercaptoethanol, as demonstrated by SDS−PAGE (data not shown).

HPLC analysis on a SP5PW cation-exchange column showed that the four RNc mutant proteins have different binding affinities to the resin in the following order: RNc-D50C > RNc > RNc-K25C > RNc-K8C > RNc-K87C. Remarkably, this order is what would be expected from the location, with respect to the enzymic interaction domain, of modifications that eliminate a positive charge (Koppenol & Margoliash, 1982). For RNc-D50C, a negative charge is removed, increasing the net positive charge, allowing the protein to bind stronger than RNc to the resin. There also was a correlation between the binding affinities of different Cc mutants to the cation-exchange resin and their binding affinities to CcP as evidenced from the apparent K_m values for the high-affinity phase of the steady-state kinetics of the reaction between CcP and various Cc. RNc-K87C had the weakest binding to the enzyme, horse or rat Cc the highest, and RNc-K25C/RNc-K8C intermediate affinities. The one exception to this correlation was the RNc-D50C mutant which bound the most strongly to the cation-exchange resin but had an apparent K_m value intermediate between those for RNc-K87C and RNc-K8C or RNc-K25C. This presumably indicates that the RNc-D50C mutation, far removed from the enzymic interaction domain, results in changes other than its effect on the net charges of the protein, the parameter that determines binding to the resin. In general, these results confirm the earlier definition of the binding domain (Margoliash & Bosshard, 1983) and show that, as observed with the 4-carboxyl-2,6-dinitrophenyl (CDNP) derivatives of horse Cc (Kang et al. 1978), the binding affinities of the various mutants to a cation-exchange resin change in roughly the same order as those with CcP.

In the case of the three SCc mutants, though they are all located in the enzymic interaction domain, one removes a positive charge (SCc-K87C), while the other two involve uncharged residues (SCc-G83C and SCc-V28C). This is again reflected in their binding affinity to the cation-exchange resin and to CcP, with SCc-K87C showing the weakest binding, while SCc-G83C and SCc-V28C exhibit binding affinities similar to that of SCc.

APB-Modified Mutant Cc. The UV−visible spectra of APB-modified mutant Cc showed an increased absorbance at 300 nm resulting from the bound azidophenacyl moiety, without any changes from 400 to 750 nm. This made it possible to determine spectrophotometrically the molar ratio of the arylazido group to Cc, which varied from 0.8 to 1.0 for many experiments with various mutant Cc. Analysis of these products by reversed-phase HPLC showed an increased binding affinity to the resin as expected from the addition of the extra hydrophobic group to the protein, with retardation of about 5 min, over total run times of 60 min, as compared to the unmodified mutant proteins. By contrast, cation-exchange HPLC showed minor or no changes in the binding affinity of the arylazido-modified proteins, also as expected since the modification of cysteine involves no change in charge. With both chromatographic procedures, all the derivatized mutant proteins yielded a single sharp peak indicating that a homogeneous product had been obtained, with the APB covalently attached.

In the present study, since multiple 1:1 covalent complexes between Cc and CcP are generated for any particular Cc (see below), it is essential to employ homogeneous arylazido-Cc. Otherwise it would be impossible to decide whether such multiplicity reflects multiple interactions of Cc with

the enzyme or merely the binding through different residues of C*c*. Furthermore, any work to determine the locations of covalent binding on C*c*P would be substantially more difficult.

Cross-Linking Cc to CcP. The results of cross-linking experiments were assayed by SDS−PAGE. Following UV irradiation, three new species appear with apparent molecular weights corresponding to those of 2:1 C*c* to C*c*P complex, 1:1 C*c* to C*c*P complex, and dimeric C*c* (see Figure 2, lane 1), while C*c*, C*c*P, and a mixture of C*c*P and C*c* controls showed only their initial components. The proportion of the three new species increased with irradiation time up to 30 min and were clearly not the result of disulfide dimerization since they remained stable under the reducing conditions of the denaturing gel. It should be noted that in covalent complexes formed with a bifunctional reagent, such as EDC, both 1:1 and small amounts of 2:1 C*c*−C*c*P covalent complexes have also been observed (Waldmeyer & Bosshard, 1985; Moench et al., 1993).

In attempting to maximize the yield of 1:1 covalent complex, the RN*c*-K87C mutant was employed to test the influence of buffer ions (Tris−chloride, Tris−acetate, sodium phosphate, sodium acetate), pH (5.0−9.0), ionic strength (close to 0 to 100 mM), exposure time to UV light (0−64 min), the concentrations of the proteins (1−100 μM), and the molar ratio of C*c*P to C*c* (1.1 to 0.1). The buffer ions had only small effects; pH values of 8 and above decreased the yield of 1:1 covalent complex dramatically; UV light exposure beyond 30 min did not substantially affect the yield; increasing either the absolute or the relative concentration of C*c* merely resulted in increasing the amount of covalent C*c* dimer with little effect on the yield of 1:1 covalent complex. By far the most important parameter was found to be ionic strength, with the best yields at 10 mM or below, decreasing rapidly with increasing ionic strength. The optimal conditions for the formation of 1:1 covalent complex defined in this manner are those given for its preparation (Materials and Methods) and were found to be those that favor the high-affinity site interaction between C*c*P and C*c* (Kang et al., 1977).

It was found that the higher the binding affinity of the C*c* mutant for C*c*P and the more centrally located the cross-linker with respect to the enzymic interaction domain of C*c*, the higher the yield as judged by SDS−PAGE (data not shown). Thus, as expected from the relative binding affinities of mutant C*c* (see above) and of fungal vs mammalian C*c* to C*c*P (Kang et al., 1977; Kim et al., 1990), the mutant SC*c*-V28C generated more 1:1 covalent complex than SC*c*-K87C, though they both are located within the enzymic interaction domain of C*c*. These two SC*c* mutants generated larger amounts of 1:1 covalent complex than the RN*c*-K87C mutant which is also located in the interaction domain. Furthermore, the RN*c*-K87C mutant produced more complex than the mutants RN*c*-K8C and RN*c*-K25C which are peripherally located in the interaction domain, while with the RN*c*-D50C mutant which is located on the "bottom" surface of the protein and entirely away from the interaction domain, 1:1 complex could only be detected in the reaction mixture after a 1000-fold concentration. These correlations between the yields of the 1:1 complexes and the binding affinities of the mutant C*c* as well as the locations of the cross-linking group indicate that the 1:1 covalent complexes formed are most likely the result of interactions between the enzymic interaction domain of C*c* and the high-affinity site of C*c*P.

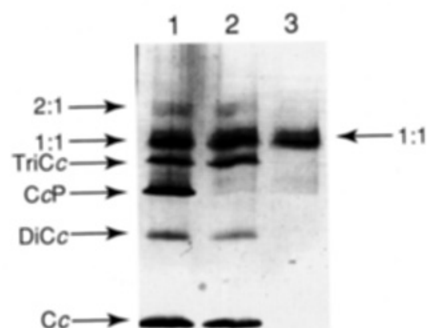

FIGURE 2: Purification of the 1:1 covalent complex SC*c*-K87C−C*c*P followed by SDS−PAGE. A 12.5% homogeneous SDS gel for the Pharmacia PhastGel system was used. Electrophoresis and silver-staining were carried out according to manufacturer's instructions. *Lane 1*, cross-linking mixture. *Lane 2*, cross-linking mixture after DEAE-Sepharose absorption. *Lane 3*, cross-linking mixture after adsorption on both DEAE- and CM-Sepharose. The positions of C*c* monomer (C*c*), C*c* dimer (DiC*c*), C*c* trimer (TriC*c*), C*c*P, 1:1 C*c*−C*c*P covalent complex (1:1), and 2:1 C*c*−C*c*P covalent complex (2:1) are marked. Note that the band for the 1:1 C*c*−C*c*P covalent complex is relatively diffuse and the band for the 2:1 C*c*−C*c*P complex is very faint.

Purification of 1:1 CcP−Cc Covalent Complex. The 1:1 covalent complexes of C*c*P with both RN*c* and SC*c* mutants were purified as described (Materials and Methods), the procedure being followed by SDS−PAGE (Figure 2). Lane 1 shows the cross-linking mixture, containing free C*c*, C*c* polymer, free C*c*P, 1:1 C*c*−C*c*P covalent complex, and a small amount of 2:1 C*c*−C*c*P covalent complex. Lanes 2 and 3 are the solutions after passage through the DEAE-Sepharose column and CM-Sepharose column, respectively. As expected, the DEAE-Sepharose removed free C*c*P and the CM-Sepharose free C*c*, C*c* polymers, and 2:1 complex, leaving the 1:1 complex sufficiently pure for kinetic studies. Spectroscopic assay of this purified material indeed showed that the ratio of C*c*P to C*c* was 1.

A remarkable result was the finding that the purified 1:1 C*c*-C*c*P covalent complex consisted of a mixture of several chromatographically different species. For example, in Figure 3, more than seven different complexes are visible. This multiplicity was originally seen in the SDS−PAGE analysis (see Figure 2), and confirmed by isoelectric focusing and electrophoretic titration curve experiments with 1:1 complexes of both RN*c* and SC_c mutants which had several different bands for the 1:1 covalent complex (data not shown). A similar phenomenon was observed with SDS−PAGE by Moench et al. (1993) with 1:1 SC*c*−C*c*P covalent complex but not with horse C*c*-C*c*P complex. However, the most direct demonstration of this multiplicity came from cation-exchange HPLC of the purified 1:1 covalent complex which showed multiple peaks (Figure 3A), and the protein in each chromatographic peak migrated to the same position corresponding to the 1:1 covalent complex on SDS−PAGE (data not shown). This demonstration of the formation of several 1:1 complexes, each of which differs slightly in its binding to the cation-exchange resin, is strong support for the concept that the C*c*−C*c*P interaction allows for mobility of C*c* among a variety of slightly different sites on the surface of the enzyme at the so-called high-affinity interaction domain (Hoffman & Ratner, 1987; Northrup et al., 1988; Hoffman et al., 1990). Furthermore, in all such chromato-

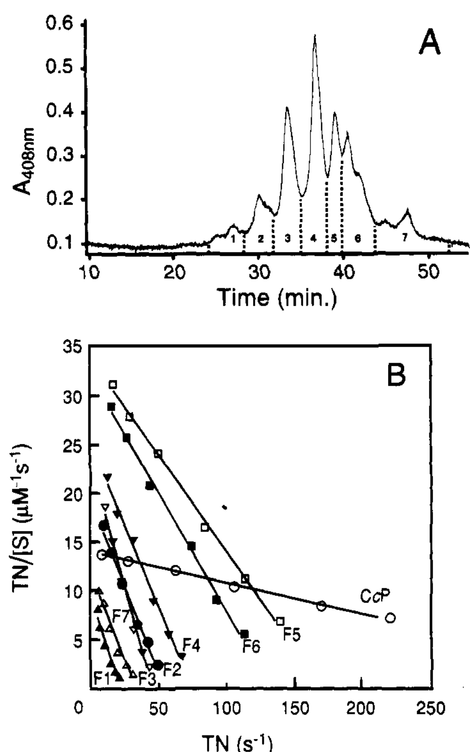

FIGURE 3: Chromatographic separation of components of the 1:1 covalent complex RN*c*-K87C−C*c*P and their enzymic activities. (A) Cation-exchange HPLC of the purified 1:1 covalent complex on a SynChropak CM300 column (25.0 × 0.46 cm). Buffer A: 10 mM potassium phosphate, pH 5.5. Buffer B: buffer A containing 400 mM KCl. Flow rate: 1 mL/min. Linear gradient from 0% B to 15% B at a rate of increase of 1/3% per minute. Seven fractions were collected as indicated in the figure. (B) Comparison of the peroxidase activities of different chromatographic fractions of the 1:1 covalent complex with SC*c* as substrate in 5 mM Tris−chloride, pH 7.0, buffer. The steady-state kinetics were measured spectrophotometrically as described by Kang et al. (1977). The results for the seven fractions (F_1−F_7 depicted in Figure 3A), as well as that for free C*c*P (○) under the same conditions, are presented as Eadie−Hofstee plots. The different lines are labeled with their corresponding fraction numbers. Fraction F_3 is indicated by (▽) and fraction F_7 by (△).

grams, there are one or two much more prominent chromatographic peaks than others, suggesting that some localizations of C*c* in the high-affinity domain of C*c*P lead to more stable complexes.

Steady-State Kinetics of Reaction of 1:1 Cc−CcP Covalent Complex with Cc. The steady-state kinetics of reaction of C*c*P with C*c* are well-known to be affected by the type of C*c* employed, mammalian or fungal, and the ionic strength of the assay buffer (Kang et al., 1977; Kim et al., 1990). Thus, an examination of both factors is essential to any conclusions concerning the enzymic activity of the 1:1 covalent complex. One special condition to which the complex has been subjected to is the irradiation of UV light. However, it could be readily shown that such irradiation has no effect on the steady-state activity of the free enzyme with either mammalian or fungal C*c* as substrates (data not shown). Using the 1:1 covalent complex RN*c*-K87C−C*c*P as the enzyme to test the influence of a variety of buffers (potassium phosphate, potassium acetate, potassium cacodylate, Tris−chloride, Tris−acetate, MOPS chloride, MOPS cacodylate) at concentrations from 1 to 200 mM, and over a pH range of 5.0−9.0, it was found that the optimal conditions for enzymic activity were 2 mM potassium phosphate, pH 7.5, and 5 mM Tris−chloride, pH 7.0, with horse C*c* and SC*c* as substrates, respectively. These conditions were mainly used to determine the enzymic activities of various 1:1 C*c*−C*c*P covalent complexes. With the steady-state kinetic studies done using the 1:1 covalent complex containing multiple components, the resulting K_m values represent averages of the different components and of their relative proportions. Nevertheless, they provided useful characterizations of the enzymic activities of these complexes.

Comparing with C*c*P under the same conditions and with the same substrate, it was found that the activities of either RN*c*− or SC*c*−C*c*P covalent complexes were vanishingly small with horse C*c* as substrate, as shown in Figure 4A for RN*c*-K87C−C*c*P and SC*c*-K87C−C*c*P, but were significant with SC*c* as substrate (Figure 4B). To check whether this difference is due to the differences in the enzyme binding affinity of the substrate C*c* employed, SC*c* being well-known to bind C*c*P more strongly than mammalian C*c* (Kang et al., 1977), a SC*c* mutant, SC*c*-K87C, which had lost a positive charge and binds weakly to C*c*P, was employed as substrate. As shown in Figure 4C, this mutant had very little activity with the SC*c*-K87C−C*c*P 1:1 covalent complex, as was the case for horse C*c*. In contrast, a SC*c* mutant, SC*c*-V28C, which has a binding affinity with C*c*P similar to that of SC*c*, showed significant activity with the 1:1 covalent complex (Figure 4C). Thus, the 1:1 covalent complexes of both RN*c* and SC*c* mutants with C*c*P exhibit the presence of an enzymically active site of relatively low affinity to the substrate, and therefore it is only those C*c*, native or mutant, that bind strongly to C*c*P that would show any significant level of activities.

Effect of Ionic Strength on the Enzymic Activities of CcP and 1:1 Cc−CcP Covalent Complex. With a single active site available in the C*c*−C*c*P 1:1 covalent complex, the effect of ionic strength on enzymic activity with SC*c* as substrate is relatively simple. The turnover number at any particular C*c* concentration decreases and the apparent K_m value of the reaction increases as the ionic strength increases (Figure 5A). However, with both high- and low-affinity sites available on the native enzyme, the influence of ionic strength is complicated by the presence of two sites changing differently upon changes in the ionic strength. Thus, as shown in Figure 6A, with SC*c* and native C*c*P, at low ionic strength (5 and 10 mM, Tris−chloride, pH 7.0), the reaction is monophasic low affinity, indicating activity at the low-affinity site, while the substrate bound to the high-affinity site does not appreciably turn over because of the tight binding of the product (Cc^{3+}) to that site. At 50 mM Tris−chloride, the reaction becomes biphasic, as the affinity of the product (Cc^{3+}) to the high-affinity site moderates, while at 100 mM Tris−chloride, the high-affinity turnover is so large that it masks the low-affinity site turnover altogether. With a further increase to 150−200 mM Tris−chloride, the reaction becomes biphasic again as the high-affinity site rate is cut down. Therefore, it is most likely that at ionic strengths less than the optimum (100 mM Tris−chloride, pH 7.0), product dissociation at the high-affinity site is rate-limiting, while at ionic strengths higher than the optimum, substrate association at the high-affinity site becomes rate-limiting. At the optimum, these two rates are equal. At the highest ionic strength tested (200 mM Tris−chloride, pH 7.0), the low-affinity site can still be active as illustrated in Figure

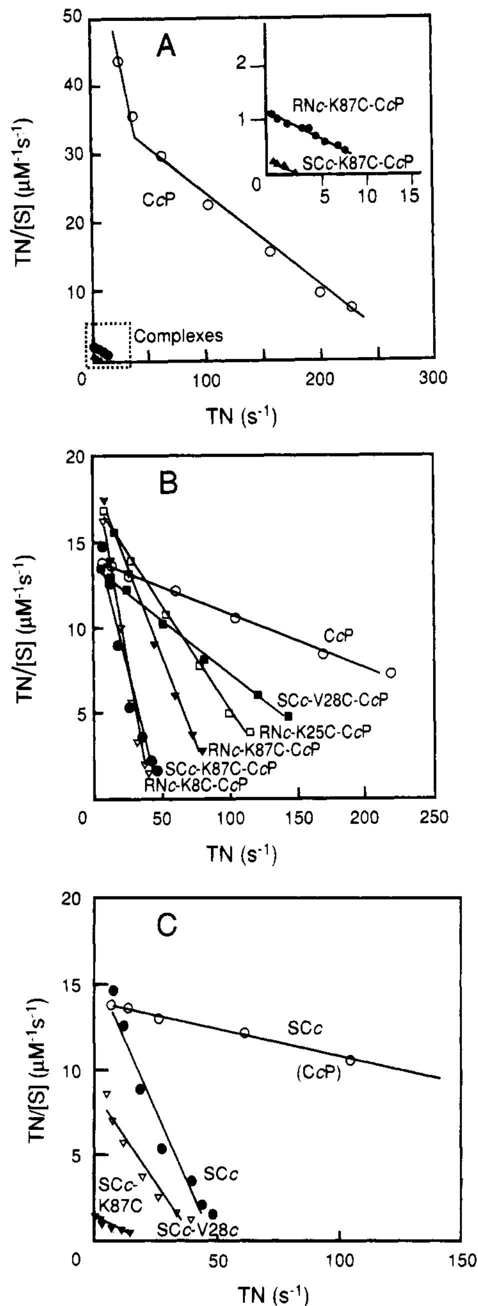

FIGURE 4: Comparison of the steady-state kinetics of free CcP and various unfractionated 1:1 Cc−CcP covalent complexes with horse Cc, SCc, or SCc mutants as substrates. The results are presented as Eadie−Hofstee plots with the identity of the enzyme indicated at each line for panels A and B, and the identity of the substrate similarly shown in panel C. (A) Horse Cc as substrate. Assay buffer: 2 mM potassium phosphate, pH 7.5. Inset: expanded view of the framed region near the origin, showing the activities of the 1:1 covalent complexes RNc-K87C−CcP and SCc-K87C−CcP. (B) SCc as substrate. Assay buffer: 5 mM Tris−chloride, pH 7.0. (C) Kinetics of reaction of SCc-K87C−CcP with SCc, SCc-V28C, and SCc-K87C as substrates. Also shown is the reaction of free CcP with SCc as substrate (O).

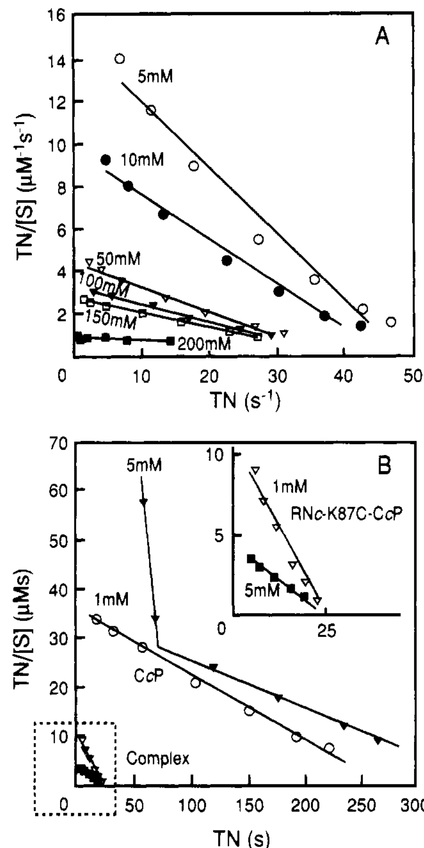

FIGURE 5: Ionic strength titration of the enzymic reaction of (A) the 1:1 covalent complex SCc-K87C−CcP with SCc as substrate and of (B) the 1:1 covalent complex RNc-K87C−CcP with horse Cc as substrate. Various concentrations of Tris−chloride, pH 7.0, buffers were used. The results are shown as Eadie−Hofstee plots, and the buffer concentrations (mM) are indicated along each line. Also shown in panel B is the reaction of free CcP with horse Cc in 1 mM (O) and 5 mM (▼) buffer concentrations. The insert shows an expanded view of the framed region near the origin.

5A which shows the activity of a 1:1 Cc−CcP covalent complex in which the high-affinity site is blocked. With the free enzyme showing much higher activity under the same conditions (Figure 6A), the activity must be mainly due to turnover at the high-affinity site.

With horse Cc, the situation is similar except that, because of weaker binding to the enzyme, any kinetic state is achieved at a much lower ionic strength than that for SCc (Figure 6B). The state that the high-affinity site of CcP is noncovaletnly blocked by tight binding of horse Cc, resulting in monophasic low-affinity kinetics, is barely reached at the lowest ionic strength tested (1 mM Tris−chloride, pH 7.0) while it was evident already at 10 mM Tris−chloride with SCc as substrate. Also to be noted is that as the assay buffer increased from 1 to 5 mM Tris−chloride, pH 7.0, the changes in the steady-state kinetics of free CcP and of 1:1 Cc−CcP covalent complex with horse Cc (Figure 5B) resemble those with SCc as substrate when the assay buffer is increased from 10 to 50 mM (Figures 5A and 6A).

Clearly, the ionic strength dependence of the kinetic behavior of both SCc and horse Cc with free CcP is characteristic of the presence of two enzymically active sites, showing different binding affinities to the substrate.

Allosteric/Electrostatic Effect. With the presence of two active sites on the enzyme, the question arises as to whether binding the substrate to one site affects the binding at the other. The present data suggest that such an effect does in fact occur. Whether this effect results from conformational changes in the enzyme as in typical allosteric effects, or merely from changes in the electrostatic field of the enzyme upon binding of the highly positively charged Cc, remains to be determined.

The observations which support such an allosteric/electrostatic effect are the following: (i) Though all 1:1

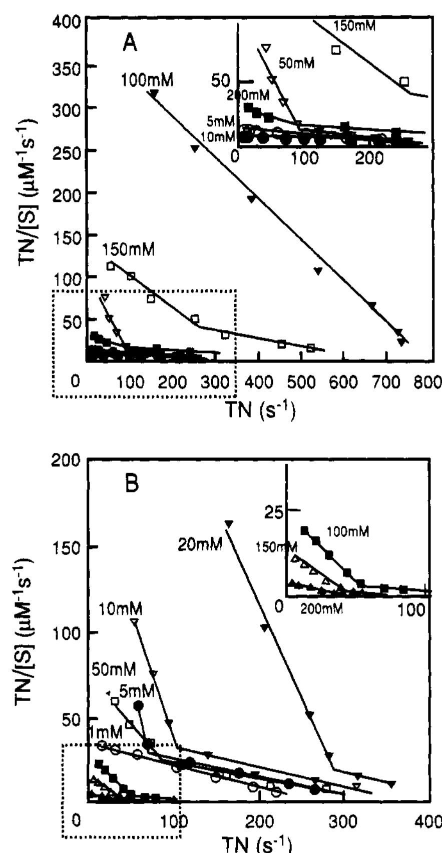

FIGURE 6: Effect of ionic strength on the reaction of free CcP with either SCc or horse Cc as substrate. Various concentrations of Tris—chloride, pH 7.0, buffers were used. The results are shown as Eadie—Hofstee plots, and the buffer concentrations (mM) are indicated at each line. (A) Free CcP reacting with SCc. The inset shows an expanded view of the framed region near the origin, containing the plots of the reactions at 5, 10, 50, and 200 mM. (B) Free CcP reacting with horse Cc. The inset shows an expanded view of the framed region near the origin, containing the plots of the reactions at 100, 150, and 200 mM.

covalent complexes of Cc with CcP prepared with different mutants of either RNc or SCc have the same low-affinity site available, they show different V_{max} and K_m values for the reaction with SCc as substrate at low ionic strength (Figure 4B). Had there been no allosteric/electrostatic effect, one would expect that the low-affinity site would have the same activity irrespective of the type of Cc bound to the high-affinity site. (ii) Even more significant, when the multiple components of the 1:1 covalent complex made with the RNc-K87C mutant were separated into several chromatographic fractions or groups of fractions, presumably cross-linked at slightly different locations, they showed a range of V_{max} and K_m values for their reactions with SCc at the low-affinity site (Figure 3B). A similar result was obtained with chromatographic fractions of SCc-K87C-CcP (data not shown). These observations imply that binding of Cc at different positions in the high-affinity site also has different effects on the binding of the substrate at the low-affinity site. (iii) The reaction of SCc with CcP at low ionic strength, at which the high-affinity site is blocked by SCc itself, exhibits only low-affinity kinetics with a K_m of 56×10^{-6} M, while the 1:1 covalent complexes, which also have the high-affinity site blocked, but by a mutant yeast or rat Cc, show kinetics with considerably lower K_m values, in the range of 2.2×10^{-6} to 15×10^{-6} M (Figure 4B). Clearly, the stronger binding SCc has a much greater allosteric/electrostatic effect than the more weakly binding mutants, even though they are fixed at the high-affinity site by a covalent bond. (iv) The reaction of horse Cc at low ionic strength (2 mM potassium phosphate, pH 7.5) with CcP is biphasic, suggesting that both sites are able to bind the substrate and turn over (Figure 4A). In contrast, after the high-affinity site is covalently blocked by a molecule of Cc, the low-affinity site could no longer react with horse Cc under the same conditions, implying decreased affinity at the low-affinity site for the substrate when the high-affinity site is occupied.

These observations indicate that Cc bound to the high-affinity site of CcP decreases the binding at the low-affinity site and that both the location of the Cc at the high-affinity site as well as its binding affinity to that site affect the magnitude of the allosteric/electrostatic effect. The stronger the binding affinity of the free Cc to the high-affinity site of CcP, the greater the decrease in binding at the low-affinity site. Thus, a more complete description of this phenomenon will have to consider both the affinities of the Cc molecules employed and the precise locations of binding on the enzyme surface.

To further test this model, SCc-V28C was prepared. It has the same charged groups as the native protein and thus an only slightly lower binding affinity to the high-affinity site of the enzyme as mentioned earlier. As expected, the 1:1 covalent complex made with SCc-V28C showed a K_m value (15×10^{-6} M) for the reaction with SCc at low ionic strength that is intermediate between that for the free enzyme (56×10^{-6} M) and those for 1:1 covalent complexes of CcP with SCc-K87C, RNc-K87C, RNc-K8C, and RNc-K25C [$(2.2-7.9) \times 10^{-6}$ M] (Figure 4B). This provides strong support for the above model, as once again the binding affinity to the high-affinity site would appear to control the magnitude of the allosteric/electrostatic effect directed to the low-affinity site.

DISCUSSION

It is generally accepted that electron transfer between CcP and Cc involves complex formation betwene the two proteins. However, whether the stoichiometry of Cc to CcP in such a complex is 1:1 or 2:1 has been controversial for nearly 2 decades. During that time, most of the experimental data favored 1:1 stoichiometry; however, the possibility of 2:1 stoichiometry was not completely ruled out. In fact, the recent study of Stemp and Hoffman (1993) provided strong evidence that at low ionic strength CcP binds two molecules of Cc, one with high affinity and one with low affinity. Furthermore, for both horse and fungal Cc, the rate of the triplet-state (ZnP)CcP → Fe^{3+} Cc electron trnasfer reaction at the low-affinity site on (ZnP)CcP is far greater than at the high-affinity site. The present results demonstrate that the high-affinity binding domain on the enzyme has multiple sites for the interaction with Cc, that the 1:1 Cc—CcP covalent complex has significant enzymic activity with SCc at low ionic strength, and that the binding of Cc to the high-affinity site decreases the binding of Cc to the low-affinity site, with different Cc mutants and different binding locations in the high-affinity domain having quantitatively different effects.

Multiple Cc Interaction Sites in the High-Affinity Binding Domain. Brownian dynamic simulations of the complexation

between C*c*P and C*c* (Northrup et al., 1988) had led to the suggestion that there are multiple interaction sites in an area on the enzyme surface which apparently yields stable electrostatic complexes (Northrup & Thomasson, 1992). A similar concept that there are multiple bound forms of the enzyme–substrate 1:1 complex, with widely different electron transfer reactivities, was developed from an examination of the kinetics of electron transfer between Cc^{2+} and (ZnP^{+})-C*c*P in the complex of the two proteins (Wallin et al., 1991).

A more direct indication of such multiplicity is provided by studies of covalently cross-linked complex of C*c* with the enzyme. Thus, Moench et al. (1993) observed by SDS–PAGE several forms of the 1:1 covalent complexes of C*c*P and SC*c* cross-linked with a carbodiimide reagent, but only a single form with horse C*c*. In the present experiments, the proteins were cross-linked at a modified residue in the enzymic interaction domain of C*c*. In all cases, the 1:1 covalent complexes obtained, employing either RN*c* or SC*c*, consisted of at least seven different forms, as estimated from their partial chromatographic separations. From their enzymic activities, they all have the C*c* bound at the high-affinity site of C*c*P. If C*c* can readily move among a number of sites on the enzyme, making the formation of an effective electron transfer complex result from two-dimensional rather than three-dimensional diffusion, this reduction in dimensionality could make the enzymic reaction process much more efficient (Northrup et al., 1988).

Other lines of evidence have also been taken to indicate that there is more than one site of binding for C*c* on C*c*P (Hazzard et al., 1988a,b; McLendon et al., 1991; Hake et al., 1992). A final determination of whether there are indeed such multiple sites for C*c* on C*c*P, rather than the classical single substrate binding site (Poulos & Kraut, 1980; Pelletier & Kraut, 1992), will probably require a much more precise definition of the high-affinity binding domain than currently available. Such data are likely to come from the several studies now under way of site-directed mutants of C*c*P (Corin et al., 1991, 1993; Choudhury et al., 1992; Hake et al., 1992; Vitello et al., 1992, 1993; Erman et al., 1993; Goodin & McRee, 1993; Roe & Goodin, 1993; Hahm et al., 1994).

Stoichiometry of the Cc–CcP Complex. As described in the introduction, whether C*c* forms only a 1:1 or also a 2:1 electron transfer competent complex with the enzyme has been extensively investigated without reaching a consensus. In the present study, cysteine mutants of both RN*c* and SC*c* were covalently bound to C*c*P *via* an arylazido moiety following activation by UV irradiation. It was shown that this irradiation by itself had no detectable effect on the steady-state kinetic behavior of the enzyme. Also, the small-sized APB is unlikely to cause significant steric hindrance in the interaction between C*c* and C*c*P and will not allow large movements of the bound C*c*. These considerations simplify the interpretation of the enzymic activities of the 1:1 covalent complexes.

The most remarkable result of this study is that the 1:1 covalent complexes of C*c* and C*c*P had very low levels of enzymic activity with horse C*c*, but significant activities with SC*c* at low ionic strength. The small activities of the 1:1 covalent complexes with horse C*c* could not be attributed to the activity of the negligible amount of free C*c*P that might exist in the samples of 1:1 C*c*–C*c*P covalent complexes, even though none could be detected by SDS–PAGE (Figure 2). Indeed, the apparent K_m values obtained with free C*c*P and the 1:1 covalent complexes are different (Figure 5B), and the residual activities of the 1:1 complexes decrease while the activity of free C*c*P increases as the ionic strength increases from 1 to 5 mM Tris–chloride (Figure 5B). Thus, both the relatively large activities of the 1:1 covalent complex preparations with SC*c* and their small activities with horse C*c* must represent the activities of the 1:1 covalent complexes themselves and are interpreted as the result of the reaction at the low-affinity active site of the enzyme. This interpretation requires that in the 1:1 covalent complex, the C*c* occupies the high-affinity site of C*c*P, inactivating it completely. Indeed: (i) The conditions employed for producing the 1:1 covalent complex (C*c*P:C*c* molar ratio of slightly over 1, protein concentrations of 2 μM) are those which favor the interaction of C*c* with the high-affinity site of C*c*P, as indicated by steady-state kinetic studies (Kang et al., 1977). (ii) The correlation between the yields of the 1:1 covalent complexes for different C*c* mutants and their binding affinities to the high-affinity site of C*c*P, as well as the localization of the mutated residue with respect to the C*c*P binding site on C*c*, indicate that, in the formation of the 1:1 covalent complex, C*c* is bound to the high-affinity site of C*c*P. (iii) Comparison of the steady-state kinetics of the reactions of free C*c*P with those of the 1:1 covalent complexes shows that in the latter the high-affinity phase has disappeared. For example, at high ionic strengths, such as 150 mM Tris–chloride, pH 7.0, the reaction of free C*c*P with SC*c* is biphasic (Figure 6A), while under the same conditions the 1:1 covalent complex, SC*c*-K87C–C*c*P, yields only monophasic low-affinity kinetics (Figure 5A). A similar situation occurs when comparing C*c*P and the 1:1 covalent complex RN*c*-K87C–C*c*P using horse C*c* as substrate at lower ionic strengths, such as 5 mM Tris–chloride, pH 7.0 (Figure 5B). Also, C*c*P shows only high-affinity phase kinetics with the RN*c*-K8C mutant at low ionic strength (2 mM potassium phosphate, pH 7.5), while the 1:1 covalent complex is completely inactive with the same mutant (data not shown). Such kinetic phenomena were observed with all the 1:1 covalent complexes formed from mutants in the enzymic interaction domain of C*c*. (iv) All the 1:1 covalent complexes with different C*c* mutants and every one of the multiple components of these covalent complexes show significant enzymic activities with SC*c* at low ionic strength; this would be very unlikely if there were only one active enzymic site, since one would expect at least some, if not all, of the complexes to have that site blocked. (v) The effect of ionic strength on the enzymic activity of the 1:1 covalent complex is monotonic as expected for a single active site, while with the free enzyme the effect of ionic strength is complex with only monophasic low-affinity kinetics occurring at very low and very high ionic strengths, biphasic high- and low-affinity kinetics appearing at intermediate values, and monophasic high-affinity kinetics at the optimal ionic strength; such complexity is very unlikely to result from the operation of a single enzymic site.

If C*c*P is considered to have only one enzymically active site, then it would be necessary for the significant activities observed with the 1:1 covalent complexes to be through the bound C*c* blocking that single active site. This is unlikely to occur, because (i) C*c* accepts and transfers electrons presumably through its front surface interaction domain containing the solvent-exposed heme edge (Margoliash &

Bosshard, 1983), and in our 1:1 Cc–CcP covalent complexes formed through the mutated residues in that interaction domain, the front surface would be buried inside the covalent complex, our of reach of a Cc molecule in free solution; (ii) Cc molecules carry strong positive charges, and the electron transfer rate between them is insignificant at low ionic strength as compared to the enzymic rate of the 1:1 covalent complex, and this rate increases with increasing ionic strength (Gupta et al., 1972), while the activity of the 1:1 covalent complex has the opposite behavior; and (iii) last, but not least, if the electron transfer were through the covalently bound Cc, one would expect little or no difference in the reactions of free and bound mammalian or fungal proteins in any combination, whereas what is observed is very low activity with horse Cc and considerable activity with SCc as substrate, corresponding to their known affinities with the enzyme and regardless of whether it is a RNc or SCc mutant that is cross-linked to CcP.

In contrast to the present results, previous 1:1 covalent complexes of CcP with Cc showed no enzymic activity (Bisson & Capaldi, 1981; Waldmeyer & Bosshard, 1985). This is probably because in one case the complex was tested with horse Cc as substrate in 10 mM Tris–acetate, pH 6, at which little or no activity can be detected due to very weak binding, and in the other, a carbodiimide reagent was used to cross-link the proteins which also modified the carboxyl groups on the enzyme (Moench et al., 1987), presumably preventing the productive interaction with the substrate Cc at the low-affinity site.

Interpretation of the Steady-State Kinetics of CcP. In light of the present results, the interpretation of the mechanism of interaction between Cc and CcP must take into account the following considerations, some of which are part of a recently proposed model (Stemp & Hoffman, 1993): (i) There are two distinct Cc binding sites on CcP, a high-affinity site and a low-affinity site. Both are able to transfer electrons; (ii) the high-affinity site contains multiple areas at which Cc may bind, and these areas are mutually exclusive; whether the same applies to the low-affinity site is not known; (iii) the binding of Cc to both sites is ionic strength dependent; (iv) an allosteric/electrostatic effect exists between these two sites, with the binding of Cc to the high-affinity site decreasing the binding affinity of Cc to the low-affinity site. Moreover, this effect varies as the Cc and its binding location in the high-affinity domain vary; (v) though SCc and horse Cc show fundamentally similar steady-state kinetics with CcP, the ionic strength dependence of these kinetics is very different because of the large difference in their binding affinities to the enzyme.

Thus, Cc will show only low-affinity steady-state kinetics at a low enough ionic strength at which the high-affinity site, tightly bound to the substrate/product, does not turn over. In such conditions, the low-affinity site activity is under the allosteric/electrostatic inhibitory effect of the Cc bound at the high-affinity site. As already described, increasing the ionic strength from this point first leads to the appearance of biphasic kinetics, followed by monophasic high-affinity kinetics resulting from a large increase in the activity of the high-affinity site masking entirely the low-affinity site activity, and then a return to biphasic kinetics as the rate-limiting step of the high-affinity phase activity changes from the "off rate" to the "on rate" of Cc. Throughout this ionic strength titration, the activity at the low-affinity site keeps decreasing. At even higher ionic strengths, both sites show little or no activity.

Clearly, the interpretation of the steady-state activity of CcP requires a complete study of the effect of ionic strength, and preferably data with both the weaker and stronger binding substrates, namely, mammalian and fungal Cc. This is particularly the case when other perturbing factors are introduced, such as the formation of covalent complex, the use of mutant forms of Cc or CcP, and chemical modification of either protein.

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