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# Glutamate as the Common Precursor for the Aglycon of the Naturally Occurring C-Nucleoside Antibiotics†

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ABSTRACT: Pyrazofurin is one of four naturally occurring C-nucleoside antibiotics; it is elaborated by *Streptomyces candidus*. The biosynthesis of the pyrazole ring of pyrazofurin has been studied by using $^{13}C$- and $^{14}C$-labeled acetate. Carbon-13 incorporation into pyrazofurin was observed by proton-decoupled $^{13}C$ Fourier transform NMR spectroscopy. The incorporation of $^{14}C$ from [1-$^{14}C$]acetate was 0.7%. The enrichment of carbons 3, 4, and 5 of pyrazofurin from [2-$^{13}C$]acetate by *S. candidus* confirms earlier findings that acetate is converted to glutamate by the combined action of the Krebs cycle and malic enzyme [Elstner, E. F., Suhadolnik, R. J., & Allerhand, A. (1973) *J. Biol. Chem. 248*, 5385]. Malic enzyme will give rise to [1,2-$^{13}C$]acetate from [2-$^{13}C$]acetate. The [1,2-$^{13}C$]acetate is then converted to glutamate labeled with $^{13}C$ in carbons 2–5. The $^{13}C$ incorporation data indicate that carbons 1, 2, 3, and 4, but not 5, of glutamate serve as the four-carbon donor for the carboxamide carbon, C-5, C-4, and C-3, respectively, of the pyrazole ring of pyrazofurin.

Four naturally occurring C-nucleoside antibiotics have been isolated from the *Streptomyces*. They are showdomycin, oxazinomycin (minimycin), formycin, and pyrazofurin [see Figure 2 for structures; for reviews, see Bloch (1978) and Suhadolnik (1979, 1981)]. These C-nucleoside antibiotics inhibit RNA and DNA synthesis in bacteria, tumor cells, and viruses without affecting the nucleotide, RNA, and DNA processes in the *Streptomyces*-producing organisms. Pyrazofurin is of special interest because it represses replication of vaccinia, herpes simplex, measles, rhino, and influenza viruses in cell culture, inhibits the formation of vaccinia tail lesions in mice, and is in current testing for the treatment of breast cancer (Descamps & De Clercq, 1978; Gutowski et al., 1975; Cadman et al., 1978a; Hill & Whelan, 1980). Pyrazofurin, after conversion to its 5′-phosphate, competitively inhibits orotidylate decarboxylase (Sweeney et al., 1973; Cadman et al., 1978b; Dix et al., 1979). The chemical syntheses of showdomycin have been reported by Kalvoda (1976) and Trummlitz & Moffatt (1973), oxazinomycin by De Bernardo & Weigele (1977), formycin by Kalvoda (1976, 1978) and Buchanan et al. (1980a), and pyrazofurin by De Bernardo & Weigele (1976) and Buchanan et al. (1980b).

The finding that UMP serves as the precursor for pseudouridine 5′-monophosphate in tRNA (Uematsu & Suhadolnik, 1973; Cortese et al., 1974), together with earlier reports from this and other laboratories on the biosynthesis of the *N*-ribosyl antibiotics in which the purine ring serves as the precursor for

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the modified purine ring in the nucleoside antibiotics (Suhadolnik, 1979), suggested that pseudouridine might be the precursor for the biosynthesis of the C-nucleoside antibiotics. In vivo experiments with radioactive uracil, uridine, and pseudouridine showed no incorporation of radioactivity into the maleimide ring of showdomycin. Subsequent studies showed that $^{3}H$-, $^{14}C$-, and $^{13}C$-labeled acetate was converted to glutamate and that glutamate (or $\alpha$-ketoglutarate) served as an asymmetric four-carbon intermediate for the biosynthesis of the maleimide ring of showdomycin by *Streptomyces showdoensis* (Elstner & Suhadolnik, 1971, 1972; Elstner et al., 1973). Similarly, the biosynthesis of the 1,3-oxazine-2,4-dione ring of the C-nucleoside, oxazinomycin, also occurs via the conversion of acetate to glutamate such that carbons 3, 4, and 5 of glutamate become carbons 6, 5, and 4 of oxazinomycin (Isono & Suhadolnik, 1977; Isono & Uzawa, 1977). Ochi et al. (1979) have subsequently reported that [U-$^{13}C$]-glutamate was incorporated into the pyrazolopyrimidine ring of formycin. Reexamination of Ochi's $^{13}C$ NMR data by Buchanan et al. (1980c) shows that C-1 through C-4 of glutamate serves as the four-carbon donor for carbons 6, 5, 4, and 9 of the aglycon of formycin. Most recently, Buchanan et al. (1980c) reported a 0.37% and 1.1% incorporation of $^{14}C$ from [1-$^{14}C$]- and [U-$^{14}C$]glutamate, respectively, into pyrazofurin by *Streptomyces candidus*. Their data strongly suggest that C-1 to C-4 of glutamate (or $\alpha$-ketoglutarate) is a specific precursor for the amide carbon and C-5, C-4, and C-3, respectively, of pyrazofurin; however, the location of the $^{14}C$ incorporated into pyrazofurin was not determined.

In the studies described here, we report on the incorporation of $^{14}C$ from [1-$^{14}C$]acetate and $^{13}C$ enrichment of carbons 3–5 of the pyrrole ring of pyrazofurin following the incorporation of [2-$^{13}C$]acetate by *S. candidus*. Glutamate (or $\alpha$-ketoglutarate) is proposed as the common precursor for the aglycon of the four known naturally occurring C-nucleoside antibiotics.

## Materials and Methods

[1-$^{14}C$]Acetate was purchased from New England Nuclear; [2-$^{13}C$]acetate was obtained from Merck and Co.

*Growth of S. candidus and Isolation of Pyrazofurin.* Cultures of *S. candidus* were obtained from Dr. R. Hamill, Eli Lilly Research Laboratories. The subcultures were maintained on nutrient agar from which agar plugs were made and maintained at −20 °C. Seed medium consisted of trypticase and soy broth. One agar plug was added into a 250-mL flask containing 50 mL of trypticase–soy broth medium. After 48 h on a New Brunswick rotary shaker (setting = 6) at 30 °C, 2-mL aliquots of the seed culture were transferred to 2-L baffled flasks containing 300 mL of medium which consisted of 1% glycerol, 0.5% soy peptone, 0.2% $Ca(NO_3)_2 \cdot 4H_2O$, and 0.3% Produlac (National Distiller's Products Co., Louisville, KY) in tap water, adjusted to pH 6.8 with sodium hydroxide. Sixty hours after inoculation, 3 mL of either [1-$^{14}C$]acetate (0.12 mmol; 24 $\mu$Ci) or [2-$^{13}C$]acetate (1800 $\mu$mol; 95% $^{13}C$) was added to each of six flasks. Pyrazofurin was isolated 12 h later. The culture broth was centrifuged, 500*g*, 10 min, and 0 °C; the sediment was washed with 250 mL of water and recentrifuged. The supernatant (about 750 mL) and washings (about 250 mL) were combined and poured onto a Dowex AG 1 X 8 acetate column (100 mL; 2.5 × 26 cm; 200–400 mesh). The column was first washed with water until the $OD_{263}$ of the effluent dropped to less than 0.3 and then eluted with 0.2 N acetic acid. Ten-milliliter fractions were collected. Pyrazofurin was eluted in fractions 7–25. These fractions were combined and evaporated to a syrup in vacuo. The syrup was dissolved in 3 mL of water and added to a Sephadex G-10 column (1 × 50 cm) and developed with water. Pyrazofurin fractions (9–14) were combined and lyophilized. The white powder was dissolved in 5 mL of methanol and centrifuged to remove insoluble material. The clear supernatant was concentrated to 1 mL in an air current. Pyrazofurin crystallized on standing at room temperature overnight. The crystalline pyrazofurin was filtered, and the crystals were washed with 1 mL each of methanol, methanol–acetone (1:1), and acetone: yield from [1-$^{14}C$]acetate experiment, 104 mg; yield from [2-$^{13}C$]acetate experiment, 120 mg; colorless needles; mp 170–171 °C (mp of authentic pyrazofurin 172–172.5 °C), with no depression of melting point. Further purity of the pyrazofurin was determined by (1) thin-layer chromatography (Avicel F; solvent, 1-butanol–ethanol–water, 5:2:3 v/v/v; $R_f$(pyrazofurin) = 0.37) or (2) paper chromatography (Whatman No. 3MM; solvent, 1-butanol–acetic acid–water, 4:1:2 v/v/v; $R_f$(pyrazofurin) = 0.33); $\lambda_{max}^{H_2O(pH\ 7.0)}$ of the crystalline pyrazofurin = 234 nm.

Table I: Enrichment of Pyrazofurin with $^{13}C$ from [2-$^{13}C$]Acetate

| carbon assignment[a] | $\delta$[b] | enrichment from [2-$^{13}C$]acetate[c] |
|---|---|---|
| amide | 164.74 | 1.0 |
| 4 | 140.48 | 1.09 |
| 3 | 133.96 | 1.99 |
| 5 | 128.20 | 1.55 |
| 1′ | 85.40 | 1.0 |
| 2′ | 82.57 | 0.98 |
| 3′ | 76.88 | 0.98 |
| 4′ | 75.99 | 1.0 |
| 5′ | 75.25 | 0.95 |

[a] Carbon designations are taken from Wenkert et al. (1973). [b] Chemical shifts are in ppm downfield from $Me_4Si$ in $D_2O$ containing dioxane as the internal reference; $\delta^{Me_4Si} = \delta^{dioxane} + 67.4$ ppm. 2500 accumulations and a recycle time of 10 s were used for each spectrum. Values reported are ±0.05%. [c] Calculated as times natural abundance by dividing intensities of individual peaks (computer calculated) in the enriched spectrum relative to average amide carbon atom intensity (which is the lowest field resonance) in the spectrum by intensities of individual peaks and the natural-abundance spectrum relative to amide intensity in the spectrum (Rinehart et al., 1974).

*$^{13}C$ NMR Analyses.* Proton-decoupled $^{13}C$ Fourier transform NMR spectra were obtained on a Varian XL-100 spectrophotometer at 25.2 MHz. Each scan followed a 90° radiofrequency pulse of 24-$\mu$s duration. The integral between successive scans (recycle time) was chosen to be sufficiently long (with respect to the longest spin–latticed relaxation time) to ensure that all resonances had their full equilibrium intensities. The amide carbon of pyrazofurin required the longest relaxation time. Chemical shifts were measured digitally with respect to internal dioxane.

## Results

Earlier studies on the biosynthesis of the naturally occurring C-nucleoside antibiotics showdomycin and oxazinomycin showed that [1-$^{13}C$]-, [2-$^{13}C$]-, and [$^{14}C$]acetate was converted to glutamate (or $\alpha$-ketoglutarate) which then served as the carbon precursor for the maleimide and oxazine ring (Elstner et al., 1973; Isono & Suhadolnik, 1977; Isono & Uzawa, 1977). In the studies described here, the incorporation of $^{14}C$ from [1-$^{14}C$]acetate (0.07 $\mu$M) into pyrazofurin by *S. candidus* was 0.7%. Based on the acetate–glutamate incorporation into showdomycin and oxazinomycin, it was possible that the incorporation of acetate into the pyrazole ring proceeded via the formation of [1,5-$^{14}C$]glutamate such that either C-1 to C-4 or C-2 to C-5 of glutamate served as the four-carbon precursor of the pyrazole ring of pyrazofurin. Because there is no

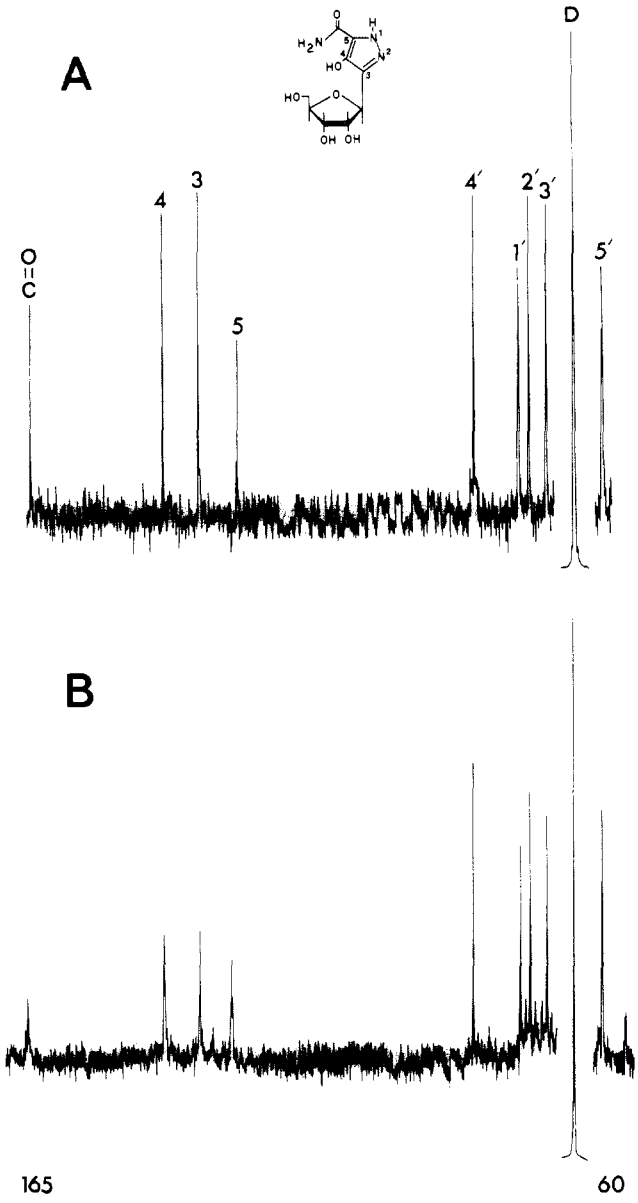



FIGURE 1: Proton-decoupled $^{13}C$ Fourier transform NMR spectra of aqueous pyrazofurin. Horizontal scale is in parts per million downfield from dioxane; 2500 accumulations and a recycle time of 10 s were used for each spectrum, at ambient temperature and 25.2 MHz. (A) Natural-abundance $^{13}C$ spectrum of pyrazofurin. Peak D is the resonance of dioxane. (B) Pyrazofurin after [2-$^{13}C$]acetate incorporation.

suitable chemical degradation of the pyrazole ring to determine the carbon atoms labeled with $^{14}C$, $^{13}C$ NMR studies were performed to determine the incorporation and distribution of $^{13}C$ following the incorporation of [2-$^{13}C$]acetate.

The proton-decoupled natural-abundance $^{13}C$ NMR spectrum of aqueous pyrazofurin (plus dioxane) is shown in Figure 1. All nine carbons are resolved into individual resonances. The $^{13}C$ enrichment spectra following the incorporation of $^{13}C$ from [2-$^{13}C$]acetate is shown in Figure 1B. These spectra were recorded with a sufficiently long recycle time to ensure no loss of intensity from partial saturation from any resonances (see Materials and Methods). $^{13}C$ enrichment into pyrazofurin from [2-$^{13}C$]acetate determined by mass spectroscopy was 4.0%.

Absorptions of individual carbons of pyrazofurin and $^{13}C$ enrichment were assigned by off-resonance decoupling experiments, standard chemical shift data, and comparison to absorptions for natural abundance of $^{13}C$ in pyrazofurin. The $^{13}C$ spectra for pyrazofurin following the incorporation of $^{13}C$ from [2-$^{13}C$]acetate showed $^{13}C$ enrichment of C-3, C-4, and C-5 of the pyrazole ring (Table I).

## Discussion

Because the ribosyl moiety of the four known naturally occurring C-nucleoside antibiotics is covalently bound to the carbon of the aglycon, several pathways could be postulated for the biosynthesis of the aglycon. In one pathway, the uracil moiety and the riboside of the C-nucleoside pseudouridine could be the precursor for the biosynthesis of the naturally occurring C-nucleoside antibiotics. In a second pathway, the biosynthesis of the aglycon would proceed via a noncarbohydrate precursor. The [$^{14}C$]- and [$^{13}C$]acetate incorporation data presented here, together with the report by Buchanan et al. (1980c), show that the biosynthesis of the aglycon of pyrazofurin proceeds via the incorporation of glutamate or the conversion of acetate to glutamate followed by the incorporation into the amide carbon and C-5, C-4, and C-3.

The present study, together with earlier reports from this laboratory (Elstner & Suhadolnik, 1971, 1972; Elstner et al., 1973; Isono & Suhadolnik, 1977) and reports by Ochi et al. (1979) and Buchanan et al. (1980c), shows that glutamate (or acetate which is converted to glutamate) is the common asymmetric precursor for the biosynthesis of the four contiguous carbon atoms of the maleimide ring of showdomycin and the pyrazole ring of formycin and pyrazofurin and the three carbon atoms of the 1,3-oxazine-2,4-dione ring of oxazinomycin (Figure 2). This conclusion is based on the incorporation and percent distribution of (1) [$^{3}H$], [$^{14}C$], and [$^{13}C$]glutamate into the aglycons of the C-nucleoside antibiotics by the *Streptomyces*-producing organisms and (2) $^{13}C$ or $^{14}C$ from [2-$^{13}C$]- or [2-$^{14}C$]acetate, following conversion to glutamate, into the carbon atoms of the aglycons of the four naturally occurring C-nucleoside antibiotics. The in vivo conversion of [2-$^{13}C$]acetate either to [2,3,4-$^{13}C$]glutamate or to [U-$^{13}C$]glutamate has been demonstrated in the *Streptomyces* that produce showdomycin and oxazinomycin (Elstner & Suhadolnik, 1971, 1972; Elstner et al., 1973; Isono & Suhadolnik, 1977; Isono & Uzawa, 1977) and in *Brevibacterium thiogenitallis* B248 (Kitano et al., 1972). The biosynthesis of the four carbons or three carbons of the aglycons of the C-nucleoside antibiotics is shown in Figure 2 and summarized as follows. C-2 to C-5 of glutamate is the four-carbon precursor for the biosynthesis of C-2, C-3, C-4, and C-5 of the maleimide ring of showdomycin; C-3 to C-5 of glutamate is the three-carbon precursor for the biosynthesis of C-6, C-5, and C-4 of the oxazine ring of oxazinomycin; C-1 to C-4 of glutamate is the four-carbon precursor for the biosynthesis of C-6, C-5, C-4, and C-9 of the pyrrolopyrimidine ring of formycin; and C-1 to C-4 of glutamate is the four carbon precursor for the biosynthesis of the amide carbon and C-5, C-4, and C-3 of the pyrazole ring of pyrazofurin (Figure 2). Furthermore, the carbon of glutamate that is covalently attached to the anomeric carbon of the ribose has been established. C-3 of the maleimide ring of showdomycin is derived from C-4 of glutamate, C-5 of the oxazine ring of oxazinomycin is derived from C-4 of glutamate, C-9 of the pyrazolopyrimidine ring of formycin is derived from C-4 of glutamate, and C-3 of the pyrazole ring of pyrazofurin is derived from C-4 of glutamate (Figure 2).

On the basis of the findings summarized in Figure 2, the biosynthesis of the naturally occurring C-nucleoside pseudouridine differs from the biosynthesis of the naturally occurring C-nucleoside antibiotics. It is now apparent that three amino

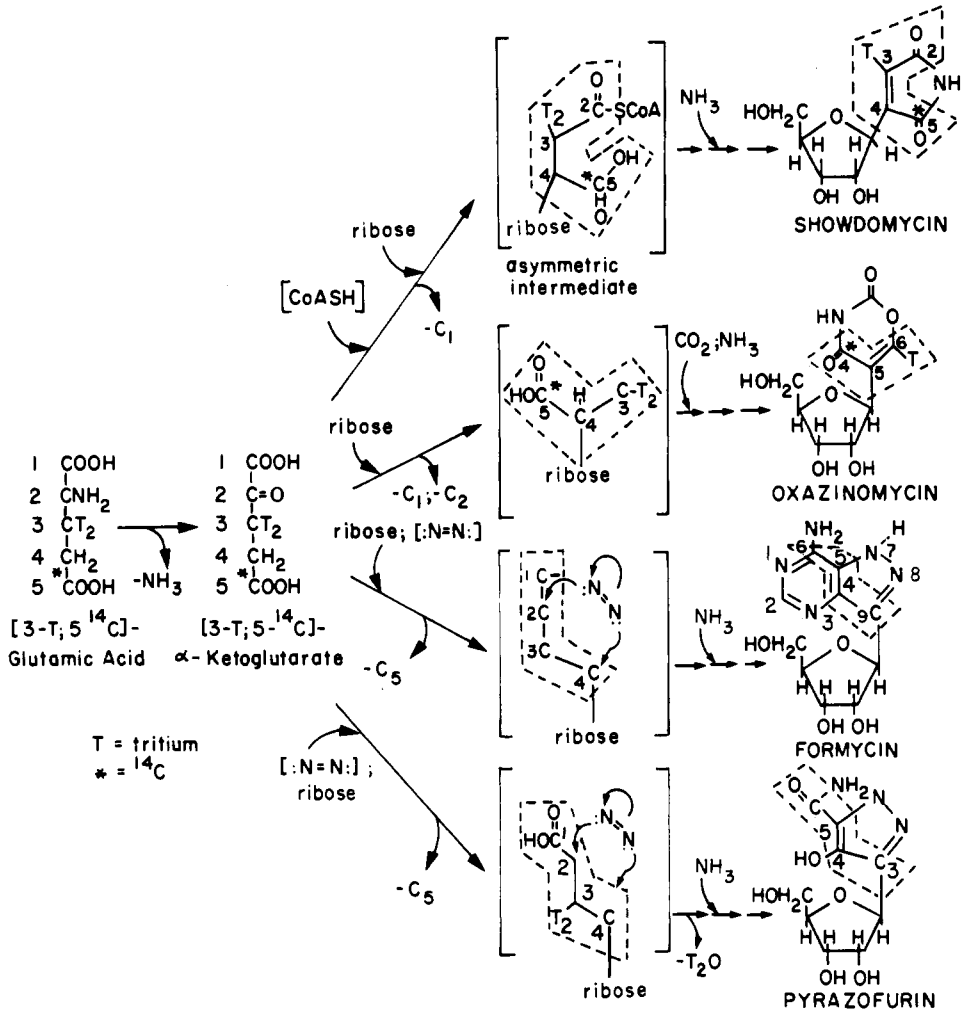
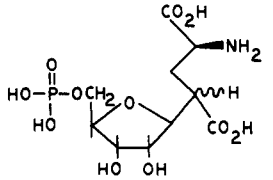



FIGURE 2: Glutamic acid as the common precursor for the carbon skeleton of the aglycons of the C-nucleosides showdomycin, oxazinomycin, formycin, and pyrazofurin.

acids are involved in the biosynthesis of the C- and N-nucleosides and nucleotides. For the purine ring, *glycine* is essential; for the pyrimidine ring, *aspartate* is essential; for the aglycon of the four known C-nucleoside antibiotics, *glutamate* is essential. The finding that glutamate is the precursor for the aglycon of the C-nucleoside antibiotics synthesized by the *Streptomyces* indicates that the biosynthesis of these nucleoside antibiotics need not compete for either glycine or aspartate which are essential for the formation of purine and pyrimidine nucleotides necessary for the energy-requiring steps in cellular processes such as RNA and DNA synthesis.

Buchanan et al. (1980c) suggest that "the biosynthesis of all the naturally occurring C-nucleoside antibiotics involves a common intermediate such as **1**, derived from ribose (as



**1**

phosphoribosyl pyrophosphate) and glutamate (or α-ketoglutarate)". Studies are under way in this laboratory with mutants of the *Streptomyces* that produce showdomycin, oxazinomycin, and pyrazofurin in an attempt to isolate **1**.

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# Covalent Complex between Yeast Cytochrome *c* and Beef Heart Cytochrome *c* Oxidase Which Is Active in Electron Transfer†

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ABSTRACT: A covalently cross-linked complex of yeast cytochrome *c* and beef heart cytochrome *c* oxidase has been prepared by the method of Birchmeier et al. (1976) [Birchmeier, W., Kohler, C. E., & Schatz, G. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4334–4338]. This complex is linked through cysteine-107 of yeast cytochrome *c* to a cysteine in subunit III of cytochrome *c* oxidase. The covalently bound yeast cytochrome *c* blocked the interaction of horse heart cytochrome *c* with the high-affinity binding site for substrate on the oxidase without affecting binding to the low-affinity site. These results along with the findings of Bisson et al. (1980) [Bisson, R., Jacobs, B., & Capaldi, R. A. (1980) *Biochemistry 19*, 4173–4178] indicate that cytochrome *c* in the high-affinity binding site occupies a cleft between subunits II and III on the cytochrome *c* oxidase complex. Covalent binding of cytochrome *c* in the high-affinity binding site inhibited cytochrome *c* oxidase activity with reduced cytochrome *c* as substrate. Complete inhibition was obtained with one cytochrome *c* covalently bound per oxidase monomer. Thus, the low-affinity site for cytochrome *c* does not function independently in electron transfer. Covalently bound cytochrome *c* was able to transfer electron from ascorbate and *N,N,N',N'*-tetramethylphenylenediamine to cytochrome *c* oxidase with an overall oxidase activity of around one-sixth of maximal. Binding of horse heart cytochrome *c* in the low-affinity site increased this electron-transfer activity to a level close to that of unmodified cytochrome *c* oxidase. Cytochrome *c* bound to the low-affinity site must increase the rate of electron transfer through the high-affinity site by some allosteric mechanism.

Cytochrome *c* oxidase (EC 1.9.3.1), the terminal member of the electron-transport chain in mitochondria, is an intrinsic membrane protein which spans the inner mitochondrial membrane. The enzyme catalyzes the transfer of electrons from cytochrome *c* to molecular oxygen while conserving the energy released in the reaction as a proton gradient for the subsequent synthesis of ATP. The cytochrome *c* oxidase monomer contains two hemes (*a* and $a_3$) and two copper atoms as prosthetic groups in a complex of molecular weight between 140000 and 170000 [reviewed in Erecinska & Wilson (1978), Capaldi (1979), and Azzi & Casey (1979)].

Three-dimensional reconstruction of two different two-dimensional crystal forms has yielded the gross shape and approximate size of the cytochrome *c* oxidase monomer (Henderson et al., 1977; Fuller et al., 1979). It is a Y-shaped structure comprising three domains, two of which ($M_1$ and $M_2$) span the lipid bilayer, with the third or C domain extending from the cytoplasmic face of the inner mitochondrial membrane (Fuller et al., 1979). Beef heart cytochrome *c* oxidase has been shown to be a dimer in solutions of many nonionic detergents (Robinson & Capaldi, 1977; Bisson et al., 1980; Rosevear et al., 1980) and in membranes (Henderson et al., 1977).

The steady-state kinetics of the reduction of cytochrome *c* by cytochrome *c* oxidase are complicated, and most models intended to explain these data invoke two cytochrome *c* binding sites per cytochrome *c* oxidase monomer (Ferguson-Miller et al., 1976, 1978; Errede et al., 1976; Smith et al., 1979; Nicholls et al., 1980). Direct binding experiments show that there is a high-affinity ($K_d \simeq 10^{-8}$ M) and a low-affinity ($K_d \simeq 10^{-6}$ M) binding site for horse heart cytochrome *c* on beef heart cytochrome *c* oxidase (Ferguson-Miller et al., 1976). Eadie–

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