In conclusion we find that the measured $E^{\circ\prime}$ values for bound cytochrome *c* differ significantly from the $E^{\circ\prime}$ for cytochrome *c* in solution. The decrease of 50 mV found in the $E^{\circ\prime}$ value when cytochrome *c* is bound makes cytochrome *c* equal-potential with the low potential heme *a* and copper components of cytochrome aa_3. It is not obvious the role this cytochrome *c* interaction with cytochrome aa_3 has in either the mechanism of electron transfer or energy conservation; however, it now appears quite certain that cytochrome *c* indeed binds to cytochrome aa_3 and each cytochrome recognizes the presence of the other.

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Use of Specific Lysine Modifications to Locate the Reaction Site of Cytochrome *c* with Cytochrome Oxidase†

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ABSTRACT: The reaction of cytochrome *c* with trifluoromethylphenyl isocyanate was carried out under conditions which led to the modification of a small number of the 19 lysines. Extensive ion-exchange chromatography was used to separate and purify six different derivatives, each modified at a single lysine residue, lysines 8, 13, 27, 72, 79, and 100, respectively. The only modifications which affected the activity of cytochrome *c* with cytochrome oxidase (EC 1.9.3.1) were those of lysines immediately surrounding the heme crevice, lysines 13, 27, 72, and 79, and also lysine 8 at the top of the heme crevice. In each case, the modified cytochrome *c* had the same maximum velocity as that of native cytochrome *c*, but an increased Michaelis constant for the high affinity phase of the reaction. This supports the hypothesis that the cytochrome oxidase reaction site is located in the heme crevice region, and the highly conserved lysine residues surrounding the heme crevice are important in the binding.

There are numerous lines of evidence suggesting that the binding of cytochrome *c* to both its reductase and oxidase is largely facilitated by ionic interactions involving positively charged lysine ϵ-amino groups on the surface of the cytochrome *c* molecule. It is not known, however, whether these lysines are involved only in favorable interactions at the binding interfaces, or whether they are also involved in the actual mechanism of electron transfer to and from the iron. Further, although recent evidence (Dickerson and Timkovich, 1975) suggests that both the reductase and oxidase bind in the general area of the exposed heme edge of the cytochrome *c* molecule, it is not clear what specific regions are involved, whether there is any overlap of the two binding sites, or whether in fact the oxidase and reductase bind to the same site on cytochrome *c*.

Selective chemical modification of single lysine residues on the cytochrome *c* molecule offers an attractive approach toward determining the involvement of these residues in the reactions with the reductase and oxidase. Dickerson and Timkovich (1975) review the effects of a number of lysine derivatives. More recently, Staudenmayer et al. (1976, 1977) reported on the preparation of five derivatives with single trifluoroacetylated lysine residues at positions 13, 22, 25, 55, and 99. In a preliminary report, Brautigan and Ferguson-Miller (1976) obtained six mono-4-carboxy-2,6-dinitrophenyl (CDNP)[1] derivatives, three of which were identified as CDNP-lysine 13, 60, and 72 cytochromes *c*.

We report here on the reaction of *m*-trifluoromethylphenyl isocyanate with horse cytochrome *c* to produce a variety of trifluoromethylphenylcarbamoyl (TFC) derivatives. Six of these have been identified as containing singly modified ϵ-amino groups at residues 8, 13, 27, 72, 79, and 100. Their characteristics, ^{19}F NMR properties, and reactivities with beef heart cytochrome oxidase are presented.

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[1] Abbreviations used are: TFA, trifluoroacetyl; TFC, trifluoromethylphenylcarbamoyl; CDNP, 4-carboxy-2,6-dinitrophenyl; Tos-PheCH$_2$Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone; TMPD, *N,N,N′,N′* tetramethyl-*p*-phenylenediamine dihydrochloride; Mops, 4-morpholinepropanesulfonic acid; TNP, trinitrophenyl; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid.

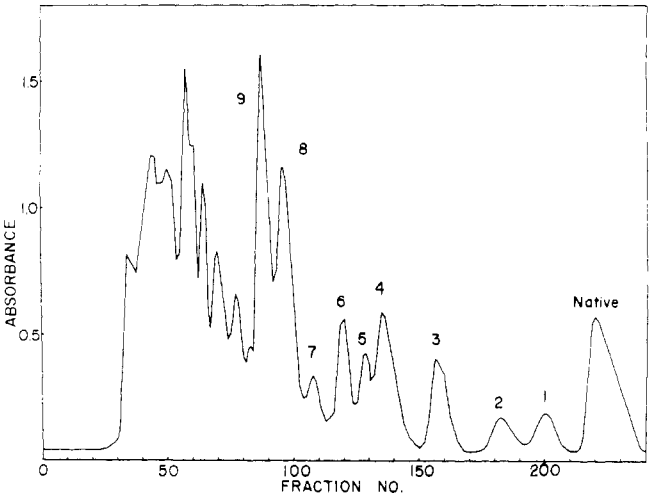

FIGURE 1: Chromatogram of 250 mg of a crude reaction mixture of TFC-cytochromes *c* on a 2 × 70 cm Bio-Rex 70 column. The column was eluted with 0.14 M ammonium phosphate, pH 7.2, at a flow rate of 25 mL/h, at room temperature. The fraction size was 4 mL. The absorbances were taken at 430 nm.

Experimental Procedure

Materials. Horse heart cytochrome *c* (type VI), deoxycholic acid, and TMPD were obtained from Sigma Chemical Co. *m*-Trifluoromethylphenyl isocyanate was obtained from PCR, Inc. Tos-PheCH_2Cl-treated trypsin was purchased from Worthington Biochemical Corp.

Preparation of Trifluoromethylphenylcarbamoyl Cytochrome c Derivatives. Horse heart cytochrome *c* (250 mg) was dissolved in 2.0 mL of 0.14 M ammonium phosphate, pH 7.2, and adjusted to pH 7.5 with 1 M NaOH. *m*-Trifluoromethylphenyl isocyanate (8 μL) was added to the rapidly stirred solution at room temperature and the pH was maintained at 7.5. After 30 min, a second 8-μL aliquot of reagent was added. After a second 30-min period, the solution was chromatographed on a small Bio-Gel P-4 column equilibrated with 0.035 M ammonium phosphate, pH 7.2, to remove unreacted reagent and side products. The cytochrome *c* was treated with ferricyanide and then applied in a narrow band to a 2 × 70 cm Bio-Rex 70 (200–400 mesh) column equilibrated with 0.14 M ammonium phosphate, pH 7.2. The column was eluted with 0.14 M ammonium phosphate, pH 7.2. The column was eluted with 0.14 M ammonium phosphate, pH 7.2, at 25 mL/h. The fractions for each peak were pooled (from quarter-height to quarter-height), concentrated on a small Bio-Rex 70 column, eluted in a small volume with 0.5 M ammonium phosphate (pH 7.2), and finally desalted by chromatographing on a small Bio-Gel P-4 column. Each peak was rechromatographed in the ferric form on a 1.6 × 7 cm CM32 column equilibrated with 0.08 M ammonium phosphate, pH 6.0. Elution was with 0.08 M ammonium phosphate, pH 6.0, at 25 mL/h. Fractions were pooled to obtain optimum purity as determined by ^{19}F NMR, concentrated as above, and desalted on small Bio-Gel P-4 columns equilibrated with buffer appropriate for further use in peptide mapping or assays.

Keilin-Hartree Preparation. Cytochrome *c* depleted Keilin-Hartree particles were prepared by the method of Smith and Camerino (1963) and stabilized with glycerol as described by Ferguson-Miller et al. (1976). Protein concentration was determined by the biuret method after solubilization of the Keilin-Hartree particles as described by Jacobs et al. (1956).

Nuclear Magnetic Resonance. ^{19}F NMR spectra were obtained at 84 MHz on a Brucker HFX 90 spectrometer interfaced with a Nicolet NMR-80 Fourier transform accessory. The internal references, trifluoroacetate and the water proton lock, were in agreement at a constant temperature of 25 °C. All samples were contained in 5-mm sample tubes.

Peptide Mapping. Rechromatographed singly modified (as determined by ^{19}F NMR) cytochrome *c* derivatives at concentrations of 1.5–5.0 mg/mL in 0.05 M Tris buffer (pH 7.5) were individually digested with Tos-PheCH_2Cl-treated trypsin (5% by weight with respect to the concentration of cytochrome *c*) at 37 °C. Hydrolysis was stopped after 3 h by freezing in dry ice followed by lyophilization. Peptide mapping was carried out on a 0.9 × 23 cm column of Aminex A-5 ion-exchange resin using a modified Phoenix amino acid analyzer according to the procedure of Staudenmayer et al. (1976, 1977). Each of the 21 peptides of the tryptic hydrolysate was identified by amino acid analysis with reference to the known primary sequence of horse cytochrome *c*.

Ascorbate–TMPD–Cytochrome c Oxidase Activity. The cytochrome oxidase activity was measured polarographically with a Gilson Model KM Clark electrode cell using the ascorbate–TMPD system (Ferguson-Miller et al., 1976). Assays were run in 50 mM potassium Mops (pH 7.5), 200 mM sucrose, 7 mM sodium ascorbate (from a fresh stock solution of 0.5 M sodium ascorbate containing 1 mM EDTA), 0.7 mM TMPD (from a fresh stock solution of 50 mM TMPD) at 25 °C. Addition of Keilin-Hartree particles (0.02 mg of protein/mL) treated with 5% deoxycholate (1 mg/mg of protein) as described by Smith and Camerino (1963) gave a low baseline rate of oxygen consumption which was subtracted from the rates of oxygen consumption measured after the addition of various amounts of cytochrome *c* (0.01 to 10 μM). The net velocities were calculated assuming 250 nmol of O_2/mL of buffer.

Visible Absorption Spectra and Redox Potential. The visible absorption spectra of the cytochrome *c* derivatives were obtained on a Cary 14 spectrophotometer. The redox potentials of the derivatives were measured by the method of Wada and Okunuki (1969).

Results

Preparation of Trifluoromethylphenylcarbamoyl Cytochrome c Derivatives. Trifluoromethylphenylcarbamoylation of cytochrome *c* was carried out under mild conditions which led to the selective modification of some of the 19 lysine ε-amino groups. The mixture of derivatives was initially separated by ion-exchange chromatography on a Bio-Rex 70 column (Figure 1). Peaks 3, 6, and 9 rechromatographed on small CM32 cellulose ion-exchange columns as single bands, while peaks 5 and 8 rechromatographed under the same conditions as two bands each (subfractions 5A, 5B, 8A, and 8B; Figure 2). Subfractions 5A, 8A, and 8B were chromatographed a second time under the same conditions. Final yields of derivatives from 250 mg of cytochrome *c* were approximately 1.0, 1.2, 3.3, 4.9, 1.8, and 7.8 mg of fractions 3, 5A, 6, 8A, 8B, and 9, respectively.

^{19}F NMR Spectra of Cytochrome c Derivatives. ^{19}F NMR spectra of rechromatographed fractions 5A, 6, 8A, 8B, and 9 each consisted of only a single resonance (Figure 3). Although fraction 3 rechromatographed as a single band, ^{19}F NMR showed that it contained a single major component and two impurities totalling approximately 20% of the fraction. However, a highly pure singly labeled derivative (Figure 3) could be obtained by pooling the subfractions from the leading half of the band only.

Peptide Mapping. The location of the TFC-lysine residue

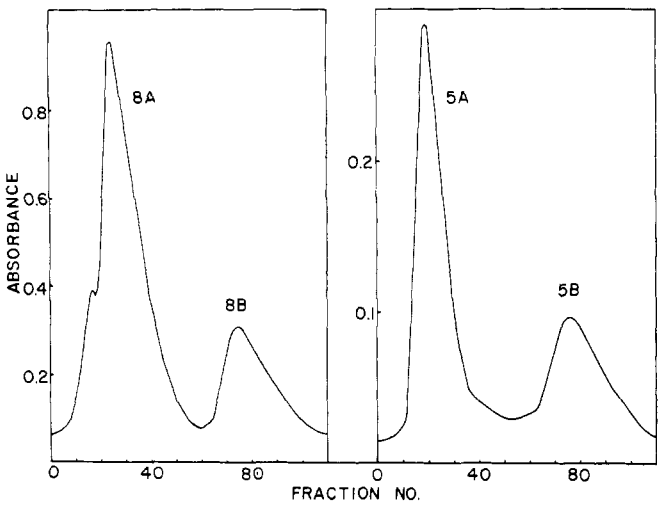

FIGURE 2: Chromatograms of TFC-cytochrome *c* fractions 8 and 5 on 7 × 1.6 cm Whatman CM32 carboxymethyl-cellulose columns eluted with 0.08 M phosphate, pH 6.0, at 25 mL/h. Fraction collection was begun after 550 mL of buffer had eluted for fraction 8, and after 950 mL of buffer had eluted for fraction 5. The fraction size was 7 mL, and the absorbances were taken at 409 nm.

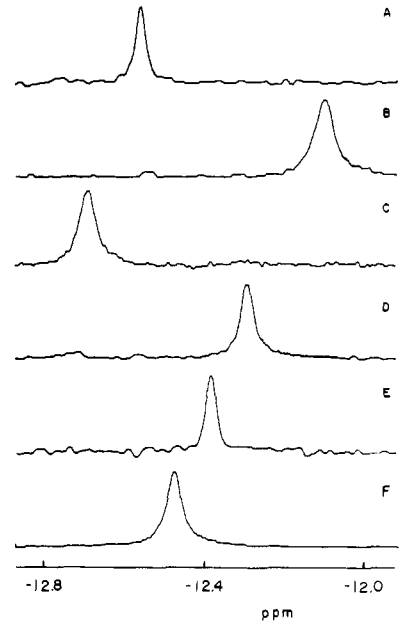

FIGURE 3: ^{19}F NMR spectra of TFC-cytochrome *c* derivatives in 20 mM sodium phosphate, pH 7.2. Each spectrum is the Fourier transform of 2000 0.5-s free induction decays. The chemical shifts are in parts per million (+ is upfield) from 10 mM trifluoroacetate in 20 mM phosphate, pH 7.2. (A) Derivative 3 (TFC-Lys-100). (B) Derivative 5A (TFC-Lys-79). (C) Derivative 6 (TFC-Lys-27). (D) Derivative 8A (TFC-Lys-72). (E) Derivative 8B (TFC-Lys-8). (F) Derivative 9 (TFC-Lys-13).

in each of the singly labeled, rechromatographed derivatives was determined by comparing the chromatograph of a tryptic hydrolysate of the cytochrome *c* derivative with a similar chromatograph of native cytochrome *c*. Trifluoromethylphenylcarbamoylation of a lysine ϵ-amino group would prevent hydrolysis by trypsin at that lysine. In the chromatograph of fraction 9, peptide 9–13 and the heme peptide 14–22 both disappeared and a new heme peptide remained at the top of the column. No other changes were observed in the peptide map with respect to native cytochrome *c*, indicating that fraction 9 contains a single modified lysine at residue 13. By the same procedure, fractions 3, 5A, 6, 8A, and 8B were shown to contain singly labeled lysines at positions 100, 79, 27, 72, and 8, respectively. Each of the singly labeled derivatives was determined to be at least 95% pure by the peptide mapping procedure and by the criterion of ^{19}F NMR (Figure 3).

Visible Absorption Spectra and Redox Potential. No detectable differences were observed between the visible absorption spectra of native cytochrome *c* and the six TFC derivatives in either redox state. The absence of any change in the conformation-sensitive 695-nm absorption band indicates that the heme environment is unmodified in the derivatives and that the samples contained no polymeric material. The redox potentials of the TFC-lysine 8 and TFC-lysine 100 derivatives were found to be identical with that of native cytochrome *c*, 260 ± 5 mV. The derivatives containing singly modified lysines at positions 13, 27, 72, and 79 exhibited slightly reduced redox potentials, 245 ± 5 mV, in each case.

Ascorbate–TMPD–Cytochrome c Oxidase Activity. The reaction of horse cytochrome *c* with beef heart cytochrome *c* oxidase was studied at cytochrome *c* concentrations (0.01 to 10 μM) for which biphasic kinetics have been observed (Ferguson-Miller et al., 1976). Native cytochrome *c* gave apparent K_m values of 4.5×10^{-8} and 7.4×10^{-7} M for the high and low affinity phases, respectively. Under these assay conditions, the apparent K_m is an empirical kinetic parameter related to binding and is to be interpreted without reference to a specific mechanism. At very low concentrations (0.01 to 1.5 μM), the TFC-lysine 8, 13, and 72 derivatives each gave monophasic kinetic plots which extrapolated to nearly the same maximal velocity, but gave substantially increased apparent K_m values compared with the high affinity kinetic phase of the native

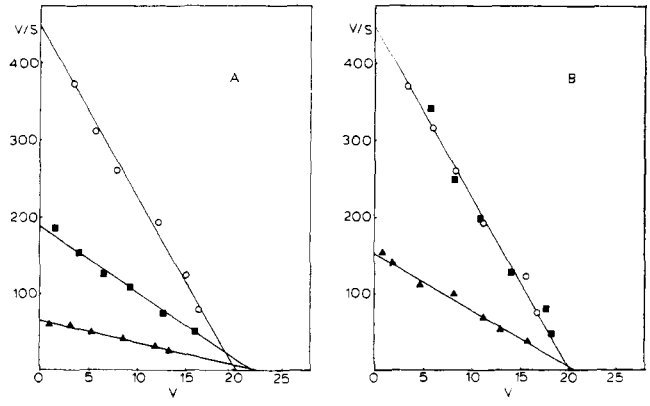

FIGURE 4: Steady-state cytochrome oxidase activities of TFC-cytochrome *c* derivatives at low cytochrome *c* concentrations (0.01 to 0.50 μM). Velocities are in nanomoles of O_2 reduced per minute. (A) Native cytochrome *c* (○); TFC-Lys-8 (■); TFC-Lys-13 (▲). (B) TFC-Lys-100 (■); TFC-Lys-72 (▲).

protein (Figure 4). Low concentrations of the TFC-lysine-100 derivative gave the same apparent low K_m and maximal velocity as native cytochrome *c* (Figure 4). The biphasic character of the kinetics of the TFC-lysine-27 and TFC-lysine-79 derivatives was much less apparent than that of native cytochrome *c*. In each case, the apparent K_m value of the high affinity phase increased and the apparent K_m value of the low affinity phase decreased while the total maximum velocity for both derivatives was about the same as that of native cytochrome *c* (Figure 5).

Discussion

Dickerson and Timkovich (1975) review a number of lines of evidence which suggest that cytochrome oxidase binds to cytochrome *c* in the general area of the heme crevice on the front face or top of the molecule, and that the interaction is probably largely ionic, involving positively charged lysine side

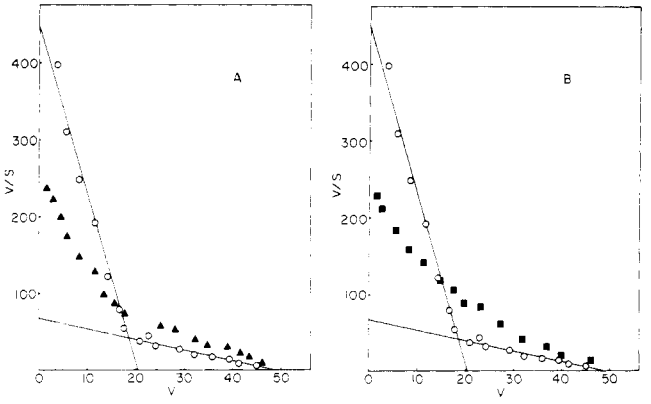

FIGURE 5: Steady-state cytochrome oxidase activities of TFC-cytochrome *c* derivatives at cytochrome *c* concentrations from 0.01 to 6.0 μM. Velocities are in nanomoles of O_2 reduced per minute. (A) Native cytochrome *c* (○); TFC-Lys-27 (▲). (B) TFC-Lys-79 (■).

chains on the cytochrome *c* molecule. Specific chemical modification of lysine ϵ-amino groups on cytochrome *c* has been used to produce derivatives in which the positive charges are replaced by negative charges, by other positive charges or simply eliminated. However, the most useful of these derivatives are those which contain a single lysine modification at a known position. Furthermore, those derivatives in which there is neither significant disruption of the molecular structure nor modification of the redox properties as a result of the side chain modification are best suited for studies of the interaction of cytochrome *c* with its reductase and oxidase.

Lysine-13 appears to be particularly susceptible to modification by a variety of reagents, to form trinitrophenyl (TNP) (Wada and Okunuki, 1969), 4-nitrobenzo-2-oxa-1,3-diazole (NBD) (Margoliash et al., 1973), 4-carboxy-2,6-dinitrophenyl (CDNP) (Brautigan and Ferguson-Miller, 1976), TFA (Staudenmayer et al., 1977), and TFC (this paper) lysine-13 derivatives. In every case, the modification of lysine-13 led to a large increase in the apparent K_m for the cytochrome *c*–cytochrome oxidase reaction with little or no change in the maximal velocity. Ferguson-Miller et al. (1976) have pointed out a remarkable similarity between the apparent K_m value of the high affinity phase measured under these conditions and the dissociation constants for the binding of cytochrome *c* to cytochrome oxidase and to cytochrome *c* depleted mitochondria. The apparent K_m values are, therefore, probably a measure of the binding of cytochrome *c* to cytochrome oxidase. While introduction of the bulky, uncharged TNP, NBD, and TFC groups gave 5- to 10-fold increases in K_m, the smaller TFA group gave a 5-fold increase and the negatively charged CDNP group produced a very large 125-fold increase. Taken together, these results suggest that removal of the positive charge at that position is more important than steric interference with binding.

In addition to the TFA-lysine-13 cytochrome *c* derivative, Staudenmayer et al. (1976, 1977) also prepared four other derivatives containing singly trifluoroacetylated lysines at positions 22, 25, 55, and 99. Of these, only TFA-lysine-25 located at the bottom right of the heme crevice affected the binding of cytochrome oxidase as reflected by a threefold increase in the apparent K_m for the high affinity kinetic phase. The fact that trifluoroacetylation of lysines 22, 55, and 99 did not affect the binding of oxidase at low cytochrome *c* concentrations is taken as evidence that various surface areas, notably the left side (Lys-55), right side (Lys-22), and back (Lys-99), of the cytochrome *c* molecule are not involved in binding to the oxidase. Furthermore, Smith et al. (1976) have presented evidence which suggests that these same areas can be ruled out.

The present studies provide additional justification for suggesting that cytochrome oxidase binds to the cytochrome *c* molecule in the area of the heme crevice. First, TFC-lysine-100 located on the back of the cytochrome *c* molecule had no effect on the binding of the oxidase. Second, the derivatives with single modifications at lysine-8 (at the top right side of the heme crevice) and lysine-72 (on the left side of the heme crevice) each resulted in a nearly threefold increase in the apparent K_m for the high affinity phase with no appreciable changes in the maximal velocity. Third, at very low cytochrome *c* concentrations, the TFC-lysine-27 and TFC-lysine-79 derivatives both exhibited significantly increased values of the apparent K_m compared with the unmodified protein. These results strongly suggest that the high affinity cytochrome oxidase binding site involves a considerable portion of the heme crevice region of the cytochrome *c* molecule. Single modifications of lysines 8, 13, 25, 27, 72, and 79 all affect the binding of the oxidase. All of the residues are found in the highly conserved positive regions surrounding the heme crevice in both bacterial and eukaryotic cytochromes *c* (Dickerson and Timkovich, 1975). This is further suggestive evidence for the involvement of these positive charges in the binding.

When the kinetic plots of the lysine-27 and lysine-79 derivatives were studied over a wide range of cytochrome *c* concentrations up to 6 μM, the total maximal velocity of the high affinity and low affinity phases was nearly the same as that of native cytochrome *c*. However, the K_m value of the high affinity phase increased and the K_m value of the low affinity phase decreased, making the biphasic character of the reaction less apparent than for native cytochrome *c*. One interpretation of these results is that lysines-27 and -79 may be at least partially involved in site discrimination. In that respect, Ferguson-Miller et al. (1976) observed large differences in the reactivities of four cytochromes *c* with beef cytochrome oxidase. In fact, one of the four, *Euglena* cytochrome *c*, with a serine at position 27 displayed no high affinity binding. It is not clear why modification of lysines-27 and -79 should increase the binding affinity of cytochrome *c* to the low affinity site of cytochrome oxidase. It is perhaps significant that the K_m value for the low affinity phase of the reaction is not very dependent on ionic strength (Ferguson-Miller et al., 1976).

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