Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Martell, A. E., and Calvin, M. (1953), The Chemistry of the Metal Chelate Compounds, New York, N.Y., Prentice Hall, p 172.
McIntosh, E. N., Mitani, F., Užgiris, V. I., Alonso, C., and Salhanick, H. A. (1973), *Ann. N.Y. Acad. Sci. 212,* 392.
McIntosh, E. N., and Salhanick, H. A. (1969), *Biochem. Biophys. Res. Commun. 36,* 552.
McIntosh, E. N., Užgiris, V. I., Alonso, C., and Salhanick, H. A. (1971), *Biochemistry 10,* 2911.
Mitani, F., and Horie, S. (1969), *J. Biochem. (Tokyo) 66,* 139.
Nagata, C., Fujita, H., and Imamura, A. (1976), *Chem. Biol. Interact. 12,* 1.
Narasimhulu, S., Cooper, D. Y., and Rosenthal, O. (1965), *Life Sci. 4,* 2101.
Oertel, G. W., and Eik-Nes, K. B. (1959), *Anal. Chem. 31,* 98.
Omura, T., and Sato, R. (1964), *J. Biol. Chem. 239,* 2370.
Raggatt, P. R., and Whitehouse, M. W. (1966), *Biochem. J. 101,* 819.
Schenkman, J. B., Cinti, D. L., Orrenius, S., Moldeus, P., and Kaschnitz, R. (1972), *Biochemistry 11,* 4243.
Schenkman, J. B., Remmer, H., and Estabrook, R. W. (1967), *Mol. Pharmacol. 3,* 113.
Schleyer, H., Cooper, D. Y., Levin, S. S., and Rosenthal, O. (1972), in Biological Hydroxylation Mechanisms, Boyd, G. S., and Smellie, R. M. S., Ed., New York, N.Y., Academic Press, p 187.
Snedecor, G. W., and Cochran, W. G. (1967), Statistical Methods, 6th ed, Ames, Iowa, Iowa State University Press, p 62.
Symms, K. G., and Juchau, M. R. (1973), *Life Sci. 13,* 1221.
Szarkowska, L., and Klingenberg, M. (1963), *Biochem. J. 338,* 674.
Tsai, R., Yu, C. A., Gunsalus, I. C., Peisach, J., Blumberg, W., Orme-Johnson, W. H., and Beinert, H. (1970), *Proc. Natl. Acad. Sci. U.S.A. 66,* 1157.
Užgiris, V. I., McIntosh, E. N., Alonso, C., and Salhanick, H. A. (1971), *Biochemistry 10,* 2916.
Užgiris, V. I., McIntosh, E. N., Graves, P., and Salhanick, H. A. (1975), in Cytochromes P-450 and b_5, Cooper, D. Y., Rosenthal, O., Snyder, R., and Witmer, C., Ed., New York, N.Y., Plenum Press, p 213.
Vore, M., Lu, A. Y. H., Kuntzman, R., and Conney, A. H. (1974), *Mol. Pharmacol. 10,* 963.

Effect of Specific Trifluoroacetylation of Individual Cytochrome *c* Lysines on the Reaction with Cytochrome Oxidase†

Nicole Staudenmayer, Siong Ng, Michael B. Smith, and Francis Millett*

ABSTRACT: We have prepared three different cytochrome *c* derivatives, each containing a single specifically trifluoroacetylated lysine at residues 13, 55, and 99, respectively. The only modification that affected cytochrome *c* oxidase (EC 1.9.3.1) activity was that of lysine-13 at the top of the heme crevice. Trifluoroacetylation of lysine-13 increased the apparent Michaelis constant fivefold compared to that of native cytochrome *c*, but did not affect the maximum velocity. Trifluoroacetylation of lysine-55 at the left side of the cytochrome *c* molecule did not affect cytochrome oxidase activity in any way, nor did trifluoroacetylation of lysine-99 at the rear of the cytochrome *c* molecule. This indicates that the cytochrome oxidase binding site on cytochrome *c* involves only the front of the cytochrome *c* molecule and those lysines immediately surrounding the heme crevice.

The mechanism by which cytochrome *c* transports electrons from cytochrome *c* reductase to cytochome oxidase in mitochondria has remained elusive despite the wealth of chemical, biochemical, and x-ray data available on cytochrome *c*. The location of the reaction sites on cytochrome *c* for cytochrome *c* reductase and cytochrome oxidase is the subject of some controversy, particularly as to whether the sites are the same or different. A number of chemical modification and antibody binding studies indicate that the binding sites might be different (Takano et al., 1973, Margoliash et al., 1973; Wilson et al., 1975), while Salemme et al. (1973) have suggested that both oxidase and reductase bind on the front of cytochrome *c* at the heme crevice and that electrons are added and withdrawn directly from the heme.

Since the binding interaction of cytochrome *c* with cytochrome oxidase is known to involve the positively charged lysines on cytochrome *c*, one way to study the location of the binding sites is to measure how modification of specific lysine groups affects the reactivity of cytochrome *c* with the reductase and the oxidase. Specific trifluoroacetylation is an attractive method because it does not appear to cause any general protein conformational changes in cytochrome *c* and the resulting derivatives can be used for ^{19}F NMR[1] studies of the binding interactions of cytochrome *c*, as well as for enzyme-kinetic studies. A change in the oxidase or reductase activities due to

† From the Department of Chemistry, University of Arkansas, Fayetteville, Arkansas 72701. *Received August 3, 1976.* This work was supported by United States Public Health Service Grant GM-20488.

[1] Abbreviations used are: TFA, trifluoroacetyl; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; TNP, trinitrophenyl; TosPheCH$_2$Cl, 1-1-tosylamido-2-phenylethyl chloromethyl ketone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Mops, 4-morpholinepropanesulfonic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; NMR, nuclear magnetic resonance.

trifluoroacetylation would indicate either that the ϵ-amino group of the unmodified lysine played some specific role in the reaction, such as electrostatic binding, or that the trifluoroacetylated lysine was oriented in such a way as to sterically interfere with the electron-transfer reaction.

We have previously reported on the preparation of two singly trifluoroacetylated cytochrome *c* derivatives containing labels at lysine-22 and -25, respectively (Staudenmayer et al., 1976). In this paper, we report on the use of a complementary procedure to prepare three additional derivatives singly trifluoroacetylated at lysine-13, -55, and -99. Their reactivities towards cytochrome oxidase, as well as their ^{19}F NMR properties are presented.

Experimental Procedure

Materials. Horse heart cytochrome *c* (type VI) was obtained from Sigma Chemical Co. Ethyl thioltrifluoroacetate was obtained from Pierce Chemical Co. TosPheCH_2Cl-treated trypsin was obtained from Worthington Biochemical Corp. Sodium cholate and TMPD were obtained from Sigma.

Trifluoroacetylated Cytochrome c Derivatives. Cytochrome *c* (250 mg in 2 ml of 0.14 M phosphate buffer) was treated with 100 μl of ethyl thioltrifluoroacetate while maintaining the pH at 10.0 by addition of 1 M NaOH with a micrometer syringe. This procedure completely trifluoroacetylated all 19 lysines of cytochrome *c* (5). Then, the solution was passed through a small Bio-Gel P-2 column to equilibrate the trifluoroacetylated cytochrome *c* with 0.15 M carbonate buffer (pH 10.7), and the solution was incubated at 25 °C for 15 h to hydrolyze all but the most stable trifluoroacetamide bonds. The solution was passed through a second Bio-Gel P-2 column to equilibrate the cytochrome *c* with 0.03 M phosphate buffer (pH 7.2) and was then applied to a 2 × 70 cm Bio-Rex 70 (200–400 mesh) cation-exchange column. 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 half-height to half-height), concentrated on a small column of Bio-Rex 70, eluted in a small volume by a buffer containing 0.5 M NaCl and 0.1 M phosphate (pH 7.0), and, finally, desalted by passing through a small Bio-Gel P-4 column equilibrated with 0.02 M phosphate buffer (pH 7.0). Each fraction was rechromatographed on Bio-Rex 70 as described above. TNP-Lys-13 cytochrome *c* was prepared according to the method of Wada and Okunuki (1969), and the identity and purity of the product were checked by peptide mapping.

Peptide Mapping. Cytochrome *c* at a concentration of 10 mg/ml in 0.05 Tris buffer (pH 7.5) was digested with TosPheCH_2Cl-treated trypsin (0.5 mg/ml) at 37 °C. Hydrolysis was stopped after 3 h by freezing in dry ice and 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 with stream splitting (Staudenmayer et al., 1976). The pyridine acetate gradients were adjusted until all of the 21 peptides of the tryptic hydrolysate of native cytochrome *c* were well resolved (Figure 3B). Each peak was identified by amino acid analysis with reference to the known sequence of horse heart cytochrome *c*. Amino acid analysis was carried out on a modified Phoenix amino acid analyzer using a 0.9 × 62 cm Durrum DC-1A resin column. Samples were hydrolyzed with 6 N HCl in evacuated sealed tubes for 24 h at 110 °C.

^{19}F Nuclear Magnetic Resonance. ^{19}F NMR spectra were obtained at 84 MHz on a Bruker HFX 90 spectrometer with a Nicolet NMR-80 Fourier transform accessory, using 5-mm NMR tubes.

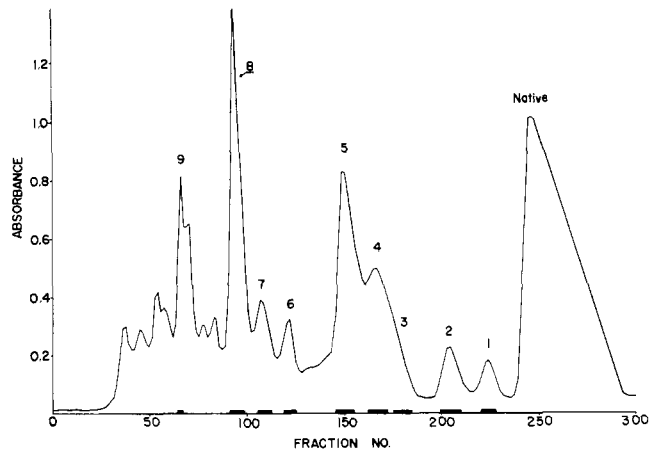

FIGURE 1: Chromatogram of 250 mg of the trifluoroacetylated cytochrome *c* preparation on a 2.0 × 70.0 cm Bio-Rex 70 cation-exchange column. The column was eluted with 0.14 M ammonium phosphate, pH 7.2, at a flow rate of 25 ml/h. The fraction size was 4 ml, and the absorbances were taken at 430 nm. Fractions were pooled as indicated by the bars along the abscissa.

Enzyme Kinetics. The derivatives used for enzyme-kinetic studies were purified on a Sephadex G-75 column and used without lyophilization to prevent formation of polymers reported by Feinberg and Brautigan (1975). The source of the cytochrome oxidase activity was a cytochrome *c* depleted Keilin-Hartree preparation prepared as described by Smith and Camerino (1963). The cytochrome oxidase activity was measured polarigraphically with a Gilson, Model KM, Clark Electrode cell using the ascorbate-TMPD system (Ferguson-Miller et al., 1976). Assays were run in 50 mM K Mops (pH 7.5), 200 mM sucrose, 10 mM sodium ascorbate (from a stock solution of 0.5 M sodium ascorbate containing 1 mM EDTA) and 1 mM TMPD. The Keilin-Hartree particles were treated with 5% deoxycholate (1 mg/mg of protein) as described by Smith and Camerino (1963). The low baseline rate of oxygen consumption measured after addition of the Keilin-Hartree particles (0.01 mg of protein/ml) was subtracted from the rates measured in the presence of various concentrations of cytochrome *c* (0.01–1.5 μM).

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

Selective trifluoroacetylation of cytochrome *c* was obtained by first treating cytochrome *c* with ethyl thioltrifluoroacetate under conditions which led to modification of all 19 lysines (Fanger and Harbury, 1965), and then hydrolyzing all but the most stable trifluoroacetamide bonds. The elution profile of the resulting mixture of derivatives is shown in Figure 1. ^{19}F NMR spectra of the pooled fractions indicated that fractions 3, 5, and 8 each consisted of a singly labeled derivative, while fraction 4 was a 60:40 mixture of two singly labeled derivatives, one of which was identical to the derivative in fraction 3 (Figure 2). Fraction 9 was a doubly labeled derivative containing the labeled lysines of both fraction 8 and 5. ^{19}F chemical shifts were obtained for each derivative in both the oxidized and the reduced states (Table I).

The location of the labeled lysine was determined by chromatographing a tryptic hydrolysate of the cytochrome *c* derivative on a cation-exchange column under conditions which

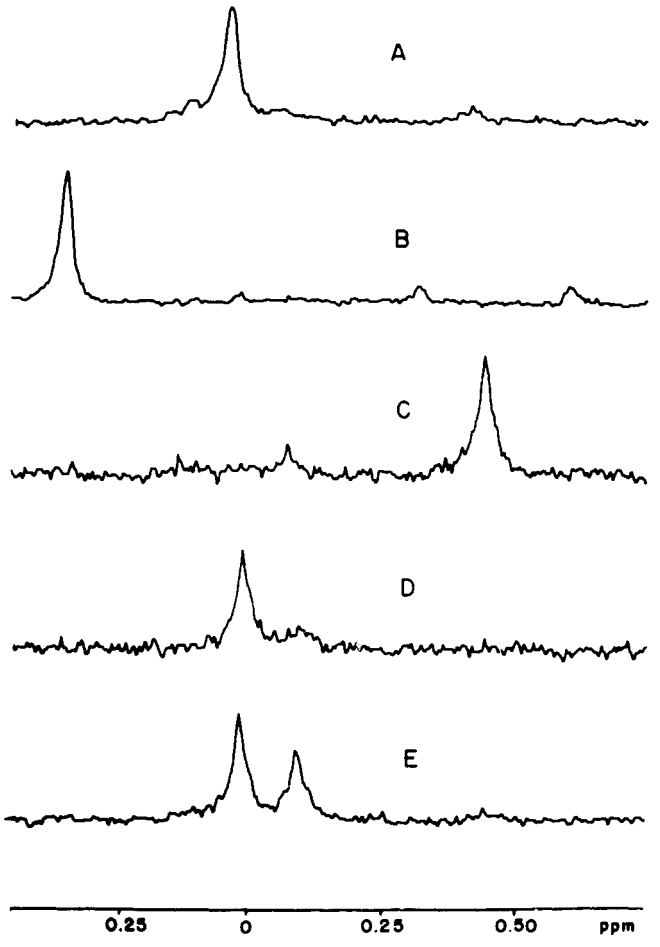

FIGURE 2: ^{19}F NMR spectra of cytochrome *c* derivatives in 0.02 M phosphate, pH 7.2. (A) 1 mM oxidized fraction 8 (Lys-13). (B) 1 mM reduced fraction 8. (C) 0.3 mM oxidized fraction 5 (Lys-55). (D) 0.3 mM oxidized fraction 3 (Lys-99). (E) 0.3 mM oxidized fraction 4. Each spectrum is the Fourier transform of 2000 2-s free induction decays. The chemical shifts are measured in ppm from 10^{-2} M trifluoroacetate in 0.02 M sodium phosphate, pH 7.2.

TABLE I: ^{19}F Chemical Shifts of Trifluoroacetylated Cytochrome *c* Derivatives.

Fraction	Modified Lysine	σ (Oxidized)[a]	σ (Reduced)[a]
3	99	−0.061	−0.124
4[b]	99	−0.061	−0.124
	?	0.043	0.061
5	55	0.377	0.560
8	13	−0.076	−0.380

[a] The chemical shifts are measured in ppm from 10 mM trifluoroacetate in 0.02 M phosphate (pH 7.2). [b] Fraction 4 contains 60% TFA-Lys-99 cytochrome *c* and 40% one other unidentified singly labeled cytochrome *c*.

led to the resolution of all 21 tryptic peptides of native cytochrome *c* (Figure 3B). The peptides were automatically detected by reaction with ninhydrin and plotted at two different absorbance scales, the one shown in Figure 3 and one fivefold more sensitive, to obtain accurate integrals of the smaller peptide peaks. The column eluent was detected at 409 nm to obtain an accurate integral of the heme peptide. The derivatives were rechromatographed on Bio-Rex 70 before peptide mapping. In the chromatogram of fraction 8, peptide 9–13 and the heme peptide 14–22 both disappeared, and the new heme peptide remained at the top of the column (Figure 3A). No other changes were observed in the peptide map, indicating that fraction 8 contains a single trifluoroacetylated lysine at residue 13. The original peptides 9–13 and 14–22 were present at less than 5% of the concentration found in the native peptide map, indicating the presence of 5% impurity, probably due to native cytochrome *c* or other derivatives. Similarly, fraction 3 was found to contain a single label at Lys-99, and fraction 5 a single label at Lys-55 (Table I). Fraction 4 contains 60% TFA-Lys-99 cytochrome *c* and 40% of one other singly labeled cytochrome *c* derivative we have not been able to identify.

TABLE II: Enzymatic Activity of Cytochrome *c* Derivatives.

Cytochrome *c* Derivative	Oxidase Activity[a]	
	K_m (μM)	V_{max} (nmol of O_2/min)
Native	0.051	27
TNP-Lys-13	0.53	26
TFA-Lys-13	0.25	27
TFA-Lys-22[b]	0.057	28
TFA-Lys-25[b]	0.14	25
TFA-Lys-55	0.051	27
TFA-Lys-99	0.046	26

[a] Measured in the ascorbate–TMPD system. [b] Staudenmayer et al. (1976).

No detectable differences were observed between the visible absorption spectra of native cytochrome *c* and the trifluoroacetylated derivatives. The absence of any change in the conformation-sensitive 695-nm absorption band indicates that the heme environment is unmodified in the derivatives. The redox potentials of the derivatives were also found to be the same as that of native cytochrome *c*, 260 mV ± 5 mV.

The cytochrome oxidase activities of the derivatives were studied using the ascorbate–TMPD system developed by Ferguson-Miller et al. (1976). None of the cytochrome *c* derivatives caused an increase in the low autoxidation rate of the ascorbate–TMPD assay solution. However, addition of Keilin–Hartree particles to the assay solution in the absence of cytochrome *c* did lead to a low baseline rate of oxygen consumption, which was subtracted from the rates measured after addition of cytochrome *c*. At cytochrome *c* concentrations from 0.01 to 1.0 μM, only the high-affinity phase of the reaction was observed and Eadie–Hofstee plots of the oxidase activity of native cytochrome c were linear with an apparent K_M value of 0.05 μM. It was found that both the TFA-Lys-13 and TNP-Lys-13 cytochrome *c* derivatives had a substantially greater K_M value than that of native, but the V_{max} values were nearly the same as that of native cytochrome *c* (Figure 4, Table II). The cytochrome oxidase activities of both TFA-Lys-55 and TFA-Lys-99 cytochrome *c* were identical to that of the native protein. We investigated the possibility that bound ions (Smith et al., 1973b) might be affecting the results. Extensive dialysis of native cytochrome *c* in the presence of Tris buffer and EDTA did not affect either the K_M or the V_{max} value of the reaction with cytochrome oxidase. Since the V_{max} values of the derivatives were identical to that of native cytochrome *c*, there were no inhibitory ions present in the derivatives (Smith et al., 1973b).

Discussion

Since the positively charged lysines on cytochrome *c* are known to play an important role in the reaction with cyto-

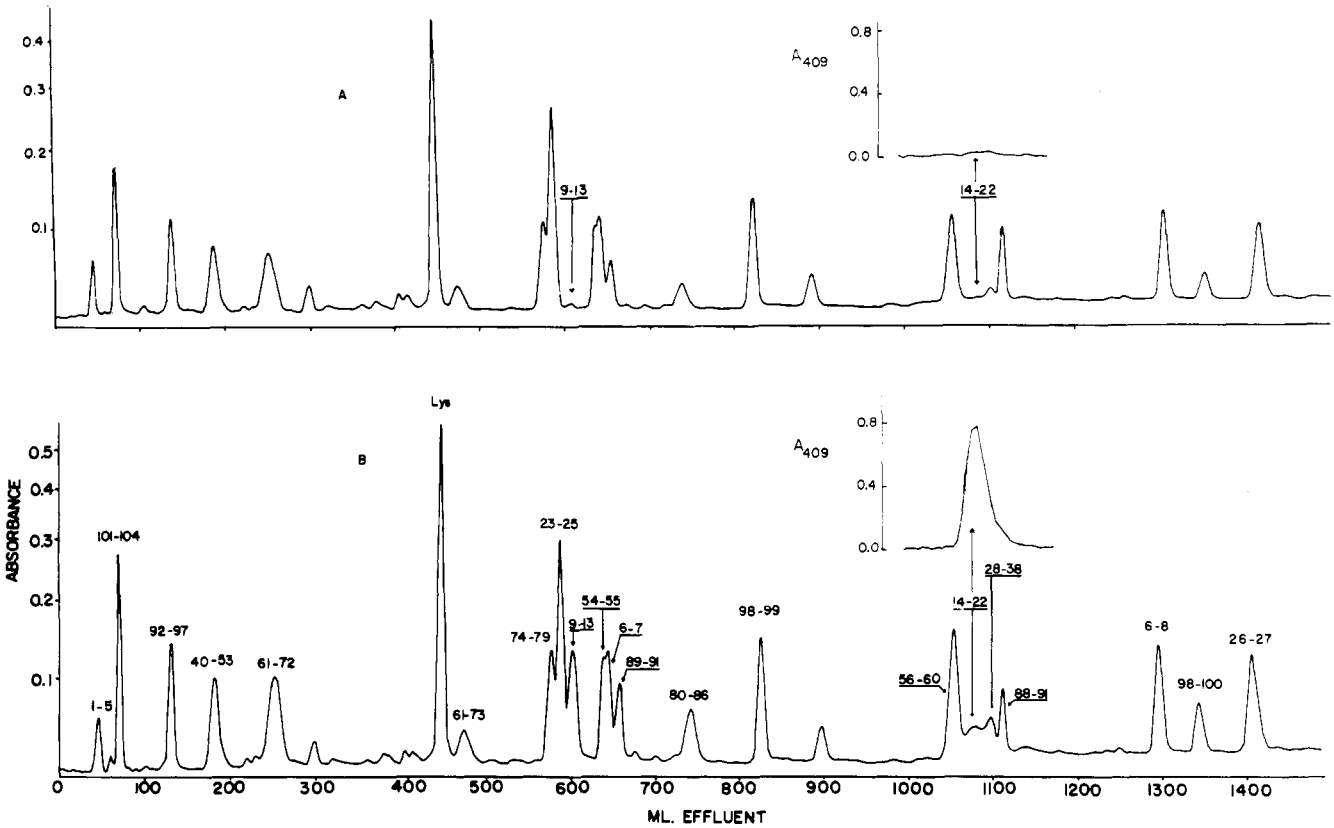

FIGURE 3: Elution profile of a 10 mg of tryptic hydrolysate of fraction 8 (A) and native horse heart cytochrome *c* (B) on a 0.9 × 23 cm Aminex A-5 column. The column was eluted first with a 1020-ml linear pH gradient from pH 3.3 (0.055 M pyridine–acetic acid buffer) to pH 4.9 (0.55 M pyridine–acetic buffer), and then with a 600-ml linear concentration gradient from the 0.55 M buffer to 3 M pyridine–acetic acid buffer (pH 5.3). The absorbances were automatically detected at 570 nm in a 3-mm flow cell after reaction with ninhydrin. The insets show the adsorbances of the column eluent at 409 nm in the region where the heme peptide elutes.

chrome oxidase, specific modification of an individual lysine with a reagent that would remove the positive charge would appear to be a rational approach towards determining the role of that lysine. Modification of Lys-13 with TNP (Wada and Okunuki, 1969) or NBD (Margoliash et al., 1973) led to a five- to tenfold increase in the apparent K_M of the cytochrome *c*–cytochrome oxidase reaction, with little or no change in V_{max}. However, since both of these groups are quite bulky, it is difficult to determine whether the effect is due to removal of the positive charge or to steric interference of the bulky group with binding. Under our assay conditions, modification of Lys-13 with the relatively small TFA group led to a fivefold increase in the apparent K_M, while modification with the bulky TNP group led to a tenfold increase. Since the bulk and hydrophobicity of these groups differ greatly from one another, it is likely that the decrease in binding strength due to trifluoroacetylation of Lys-13 is due primarily to the removal of the positive charge. Margoliash et al. (1976) have established that the apparent K_M measured under these assay conditions is nearly identical to the dissociation constant of a stable cytochrome *c*–cytochrome oxidase complex, so that the increase in the apparent K_M represents a decrease in binding affinity between the two cytochromes. Staudenmayer et al. (1976) found that trifluoroacetylation of Lys-25 at the bottom right of the heme crevice also decreased the binding affinity with cytochrome oxidase, but that trifluoroacetylation of Lys-22 on the right side of cytochrome *c* did not. The present studies indicate that Lys-55 at the entrance to the "left channel" of cytochrome *c* (Takano et al., 1973) does not play a role in the reaction with cytochrome oxidase, nor does Lys-99 at the rear of cytochrome *c*. The cytochrome oxidase binding site, therefore, involves a considerable portion of the heme-crevice region, and the positive charges of lysine-13 and -25 probably interact with negative charges on cytochrome oxidase. Since Lys-27 is located at the right side of the heme crevice close to Lys-25, it would seem logical that this group is also involved in binding, as suggested by Ferguson-Miller et al. (1976). Lys-79 at the bottom of the heme crevice and Lys-72 at the left side of the heme crevice are also possibly involved in the binding.

The rather large range in ^{19}F chemical shifts of the chemically identical trifluoroacetylated lysines is indicative of the differing environments on the surface of cytochrome *c*. In the reduced state, there will be no paramagnetic shift, and the chemical shift change, Δ, from a free trifluoroacetylated lysine will be due to the van der Waals interaction and the ring-current interaction with amino acid side chains on cytochrome *c* (Millett and Raftery, 1972). The large downfield chemical shift of the Lys-13 TFA group is probably caused by van der Waals interactions with the hydrophobic residues in the upper-heme crevice. The large upfield shift of the Lys-55 TFA group might be due to the ring-current shift of an aromatic residue in the left channel, such as Tyr-74 or Trp-59. The chemical-shift changes upon oxidation might be due to a conformational change which modifies the van der Waals interaction or the ring-current shift, or to a paramagnetic shift due to the unpaired spin on the iron. The label that experiences the least chemical-shift change upon oxidation, Lys-99, is located on the back of cytochrome *c* well removed from the heme iron. The ^{19}F NMR signals were sensitive enough to obtain spectra at concentrations down to 0.1 mM so that ^{19}F NMR

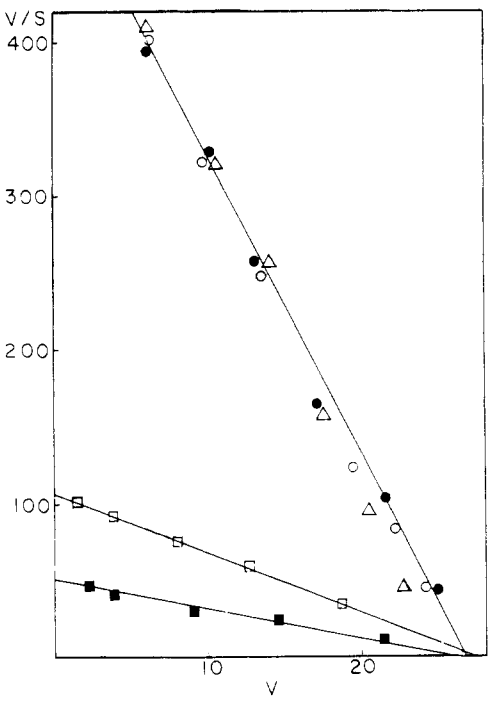

FIGURE 4: Ascorbate-TMPD cytochrome *c* oxidase activities. The velocity, v, is expressed in nmol of O_2 min^{-1}, and the cytochrome *c* concentration, S, is in μM. Activities are shown for native cytochrome *c* (●) TNP-Lys-13 (■), TFA-Lys-13 (□), TFA-Lys-55 (○), and TFA-Lys-99 (△).

studies of these derivatives bound to intact mitochondria appear to be feasible.

References

Fanger, M. W., and Harbury, H. A. (1965), *Biochemistry 4,* 2541.

Feinberg, B. A., and Brautigan, D. L. (1975), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 487.

Ferguson-Miller, S., Brautigan, D. L., and Margoliash, E. (1976), *J. Biol. Chem. 251,* 1104.

Margoliash, E., Ferguson-Miller, S., Tulloss, J., Kang, C. H., Feinberg, B. A., Brautigan, D. L., and Morrison, M. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3245.

Millett, F., and Raftery, M. A. (1972), *Biochem. Biophys. Res. Commun. 47,* 625.

Nicolls, P., Van Buuren, K. I. H., and Van Gelder, B. F. (1972), *Biochim. Biophys. Acta 275,* 279.

Salemme, F. R., Kraut, J., and Kamen, M. D. (1973), *J. Biol. Chem. 248,* 7701.

Smith, L., and Camerino, P. W. (1963), *Biochemistry 2,* 1428, 1432.

Smith, L., Davies, H. C., Reichlin, M., and Margoliash, E. (1973), *J. Biol. Chem. 248,* 237.

Smith, L., Nava, M., and Margoliash, E. (1973b), in Oxidase and Related Redox Systems II, King, T. E., Mason, H. S., and Morrison, M., Ed., Baltimore, Md., University Park Press, pp 629–638.

Staudenmayer, N., Smith, M., Smith, H., Spies, F., and Millett, F. (1976), *Biochemistry 15,* 3198.

Takano, T., Kallai, O. B., Swanson, R., and Dickerson, R. E. (1973), *J. Biol. Chem. 248,* 5235.

Wada, K., and Okunuki, K. (1969), *J. Biochem. (Tokyo) 66,* 249.

Wilson, D. F., Miyuta, Y., Erecinska, M., and Vanderkooi, J. M. (1975), *Arch. Biochem. Biophys. 171,* 108.