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Patterns of Cytochrome Oxidase Inhibition by Polycations

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Abstract

1. The inhibition of cytochrome *c* oxidase activity by three types of polycation (PL-3, PL-150 or 195, and salmine)§ is described for three kinds of oxidase system: dispersed by Tween-80, detergent-free "soluble" oxidase, and particulate oxidase (submitochondrial particles).

2. Salmine acts as a competitive inhibitor towards cytochrome *c* in all three systems, with a K_i between 1 and 4 μM. PL-3 (low M.W. polylysine) acts as a non-competitive inhibitor of oxidation in all three systems, with a K_i of between 10 μM (submitochondrial particles) and 100 μM (detergent-free oxidase).

3. PL-150 and PL-195, the high M.W. polylysines, act in three distinct ways, depending on the nature of the oxidase preparation: (a) as reversible competitive inhibitors, with K_i of about 70 nM (with oxidase dispersed in Tween-80), (b) as stoichiometric inhibitors displaying pseudononcompetitive kinetics (with Keilin-Hartree submitochondrial particles), and (c) as "superstoichiometric" inhibitors, blocking up to 100 equivalents of oxidase, cytochrome aa_3 (with detergent-free oxidase or with cholate-treated submitochondrial particles).

4. PL-195 also inhibits NADH and succinate oxidase activities in intact but *c*-deficient submitochondrial particles; sigmoidal inhibition curves can be observed in such systems. The rate of PL-195 binding was of the order of 10^6 M^{-1} sec^{-1} and the true binding constant between 0.1 and 0.01 nM for the systems showing high affinity.

5. High molecular weight polylysines may be useful in investigations of the topology and distribution of cytochrome *c* binding sites on the

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Abbreviations: PL-3, polylysine of M.W. 3000; PL-150 and 195, polylysines of M.W. 150,000 and 195,000; TMPD, N,N,N'N' tetramethyl-*p*-phenylenediamine.

mitochondrial membrane and in submitochondrial fragments and solubilized preparations. More than one oxidase molecule may be inhibited by one polymer molecule; submitochondrial fragments "opened" by cholate treatment may bind one polymer molecule to several surface sites; Keilin-Hartree particles, with a more "closed" configuration, seem to bind one polymer molecule to a single site, suggesting a "crevice" structure.

Introduction

The basic protein, salmine, and the polypeptide, polylysine, are potent inhibitors of the reaction of cytochrome *c* with soluble cytochrome oxidase [1-4]. These polycations are also effective in inhibiting the reaction of exogenous cytochrome *c* with the oxidase in Keilin-Hartree heart muscle particles and mitochondria [5].

Such observations, and others that have shown inhibition of the cytochrome *c* cytochrome oxidase reaction at high ionic strengths [6-9], have been interpreted in terms of the concept that basic cytochrome *c* (pI = 10.5, ref. 10) forms a complex with acidic cytochrome oxidase (pI = 6.0, ref. 11) in which the predominant attractive force is electrostatic in nature [8, 12, 13]. In this view, polycations are effective inhibitors because they compete with cytochrome *c* for a negative binding site on the oxidase [9]. This is however inconsistent with the finding of Takemori *et al.* [3] that polycation inhibition is noncompetitive towards the substrate cytochrome *c*.

As complex formation between cytochrome *c* and the oxidase may be an important step in cytochrome *c* oxidation both in solution and in the mitochondrion [14], we have taken another look at the inhibition by polycations using soluble cytochrome oxidase and various kinds of submitochondrial particles. The results, together with those from studies on the antibody to the oxidase [15, 16], have given some clues as to the location of the cytochrome *c* binding site. Two preliminary accounts of this work have appeared [17, 18].

Materials and Methods

Horse heart cytochrome *c*, Type III (Sigma Chemical Co.) was reduced as described previously [15]. Salmine sulfate (Grade I), poly-*l*-lysine (M.W. 3000, type II) and poly-*l*-lysine (M.W. 150,000 or 195,000, type I) were preparations of the Sigma Chem. Co. and TMPD (N,N,N′,N′ tetramethyl-*p*-phenylenediamine dihydrochloride) was obtained from Calbiochem.

Biological materials. Soluble cytochrome oxidase and Keilin-Hartree particles were prepared from beef heart as described previously [15].

Soluble cytochrome oxidase had a purity index (A 280 nm (oxidized)/A 445 nm (reduced)) of 2.09 and was solubilized in 0.1 M potassium phosphate buffer, pH 7.4, containing 1% Tween 80.

Cholate-washed particles. 10 ml Keilin-Hartree particle suspension (40 mg ml^{-1} protein) were diluted to 60 ml with 67 mM phosphate buffer, pH 7.4, plus 0.5% sodium cholate, and allowed to stand at 20° for 15 min. The particles were then collected by centrifugation (16,000 revs/min Servall head No. 34, for 30 min), washed by a second centrifugation after resuspending in a further 60 ml of 67 mM phosphate and finally resuspended in 10 ml of medium (0.58 M sucrose, 0.125 M sodium dihydrogenphosphate, 0.063 M borate, and 0.01 M EDTA, pH 7.8).

Mitochondria. Mitochondria were prepared from rat liver by the method of Chance and Hagihara [19]. Cytochrome *c*-deficient mitochondria were prepared by suspending mitochondria in 0.015 M KCl (≈10 mg ml^{-1} protein) at 0° for 10 min. The mitochondria were then centrifuged at 6000 x *g* for 10 min, resuspended in 0.15 M KCl at 0° for 10 min and subsequently centrifuged and suspended in the usual rat liver mitochondrial medium (180 mM manitol, 60 mM sucrose, 16 mM Tris-HCl, 10 mM potassium phosphate, 0.08 mM EDTA, pH 7.4), to a concentration of between 20 and 30 mg ml^{-1} (protein).

Assays. Cytochrome oxidase was assayed spectrophotometrically (Cary model II) following the oxidation of cytochrome *c* at 550 nm according to Smith and Conrad [20]. Kinetic parameters, V_{max}, K_m and K_i, were determined according to the procedures of Lineweaver and Burk [21], and of Dixon [22]. A Clark electrode (Yellow Springs Instrument Co.) was used to measure oxygen uptake. Activities are expressed as turnover number (electron equivalents per heme *a* equivalent per second). Heme *a* concentration was obtained from the difference spectrum at 605-630 nm (EmM (red-ox) = 14).

Manometric experiments were carried out with a Gilson differential respirometer system, using semimicro reaction flasks (total reaction 1.5 ml) at 30°.

Results

Reversible Binding: Detergent-dispersed Cytochrome Oxidase

Typical time courses for cytochrome *c* oxidation by a detergent-solubilized preparation of cytochrome oxidase in the presence of a polycation (PL-150) are illustrated in Fig. 1. Polycation markedly decreases the apparent first order rate constant for cytochrome *c* oxidation. In every case the molar quantity of inhibitor needed is in excess of the amount of oxidase present. No marked lag in inhibition kinetics resembling that described with lysed mitochondria [9] was seen,

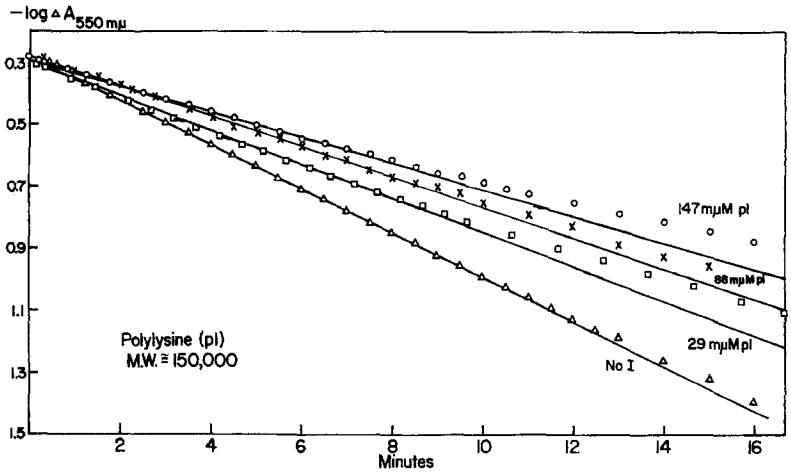


Figure 1. Time course for cytochrome *c* oxidation by soluble cytochrome oxidase in the presence of PL-150. The oxidation of ferrocytochrome *c* was followed by measuring the decrease in absorbance at 550 nm after the addition of 5.8×10^{-9} M (heme *a*) soluble cytochrome oxidase to a cuvette containing 27 μM ferrocytochrome *c* in 0.05 M potassium phosphate buffer + 1 mM EDTA + 0.1% Tween-80; pH 7.4 at 25°.

despite the absence of pre-incubation. Under these conditions the inhibitor appears to combine reversibly at single non-interacting binding sites; straight lines are obtained when 1/v is plotted against inhibitor concentration. As shown by the Lineweaver-Burk plots (Fig. 2A, B, C), salmine and PL-150 seem to act as competitive inhibitors toward ferrocytochrome *c* with K_i values of about 1.0 μM and 70 nM respectively (see Table I). On the other hand, PL-3 exhibits largely non-competitive kinetics with respect to ferrocytochrome *c*, and a K_i value between 40 and 50 μM.

Under the same conditions, bovine serum albumin had no effect on the progress of the oxidase reaction.

The maximum turnover of the oxidase was 70 electrons sec^{-1}/heme *a* equivalent and the K_m for ferrocytochrome *c* between 10 and 12 μM. The non-competitive behavior of PL-3 was confirmed by measurements of the K_m for cytochrome *c* at varying PL-3 concentrations and of the K_i for PL-3 at varying levels of ferrocytochrome *c*. The K_m for cytochrome *c* was never less than 8 or more than 12 μM in presence of PL-3.

Table I summarizes the inhibition types and K_i values observed with soluble oxidase using the spectrophotometric assay. As can be seen, the effectiveness of the inhibitors appears to be: PL-150 > salmine > PL-3.

On a residue basis, however, salmine is a slightly more effective inhibitor than PL-150 in the present assay system.

TABLE I. Polycation effects with cytochrome oxidase in presence of non-ionic detergent

Polycation	M.W.	Pattern of inhibition	K_i[a]	[E]/4K_i[b]
Salmine	5,000	Competitive	≈1 μM	3×10^{-3}
PL-150	150,000	Competitive	70 nM	3×10^{-2}
PL-3	3,000	Non-competitive	50 μM	7×10^{-5}

[a] K_i values for the spectrophotometric assay summarized below are values obtained from Dixon and Lineweaver-Burk plots.

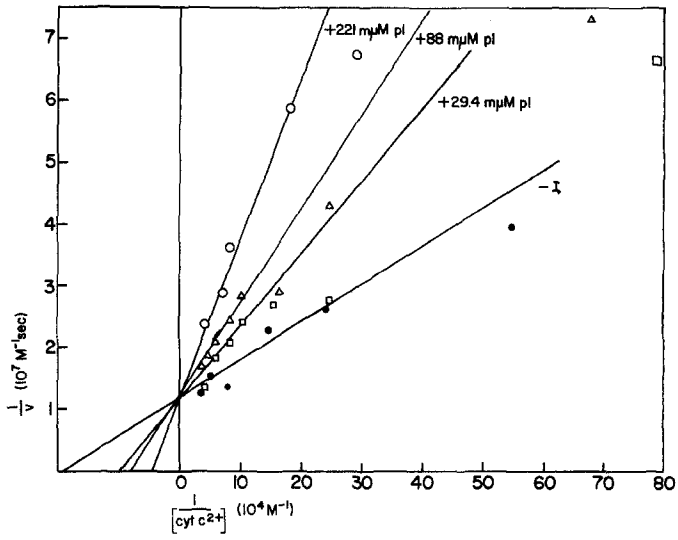
[b] E = concentration of cytochrome oxidase in heme *a* molarity; 9.3 nM heme *a* in analyses with PL-3 and salmine, and 7.4 nM heme *a* with PL-150; pH 7.4, 50 mM potassium phosphate buffer plus 1 mM EDTA, and 0.1% Tween-80, 25°.

Stoichiometric Binding: Submitochondrial Particles

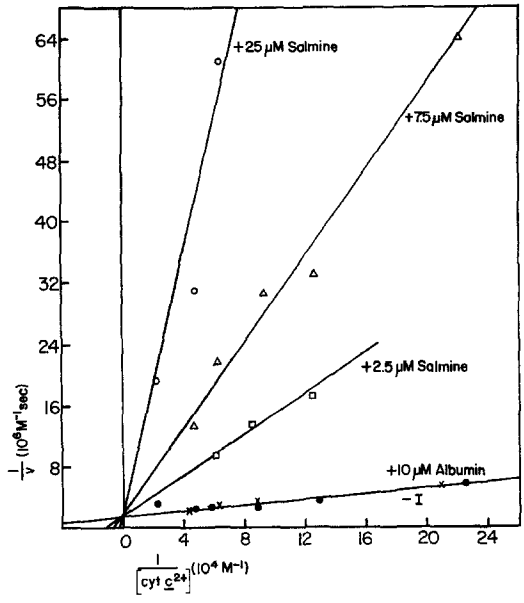
The relatively tight binding of the high molecular weight polylysine, PL-150, suggested its use to study cytochrome *c* binding sites in more complex systems. With Keilin-Hartree submitochondrial particles catalyzing ascorbate-TMPD oxidation in the presence of 25 μM cytochrome *c*, polarographic experiments showed 50% inhibition at PL-150 concentrations between 10^{-9} and 10^{-10}M.

Although salmine and low M.W. polylysine had very similar inhibitory effects to those found in the detergent-dispersed oxidase (Table I), the apparent binding of PL-150 is thus much tighter. Salmine inhibition also remained competitive ($K_i \approx 1$ μM), and PL-3 inhibition non-competitive (K_i between 10 and 20 μM). The inhibition by PL-150 in this system was, however, relatively insensitive to cytochrome *c* concentration. That is, apparent non-competitive kinetics were observed.

Further experiments showed that this pattern of inhibition is due to quasi-irreversible polycation binding to the available binding sites. Figure 3 illustrates the variation of respiration rate with increasing particle concentration in the presence of fixed amounts of polycation. As with cytochrome *c* binding [14], the particles titrate the polycation until a certain point above which the remaining oxidase is uninhibited. Under the conditions of Fig. 3, as indicated in the inset, 1 mole PL-195 titrates 1.3 moles oxidase (heme *a* equivalents) or 0.65 moles cytochrome a_3. The final slope of the plot is dependent on cytochrome *c* concentration, but the titration point is independent of cytochrome *c*. Only at very high *c* concentrations (~100 μM) or very low polylysine concentrations does the "sharpness" of the titration begin to decrease.


+221 mμM pl
+88 mμM pl
+29.4 mμM pl
-I
1/v (10⁷ M⁻¹sec)
1/[cyt c²⁺] (10⁴ M⁻¹)

(A)


+25 μM Salmine
+7.5 μM Salmine
+2.5 μM Salmine
+10 μM Albumin
-I
1/v (10⁶M⁻¹sec)
1/[cyt c²⁺](10⁴ M⁻¹)

(B)

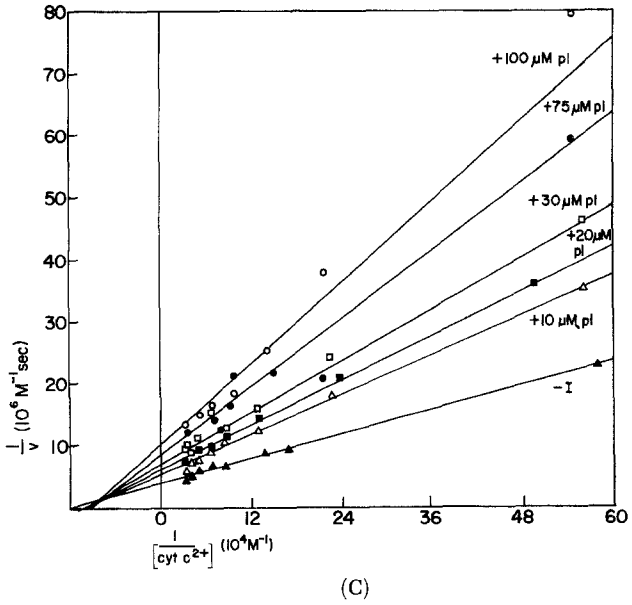


Figure 2. Kinetics of polycation inhibition. (A) High M.W. poly-*l*-lysine (M.W. 150,000). The conditions are the same as described in Fig. 1. Cytochrome oxidase (5.8×10^{-9} M heme *a*) was added to a cuvette containing ferrocytochrome *c* and 29.4 mμM PL-150 (□); 88 mμM PL-150 (△); 221 mμM PL-150 (○); or without inhibitor (○), –I. (B) Salmine. Cytochrome oxidase (9.3×10^{-9} M heme *a*) was added to a cuvette containing ferrocytochrome *c* and salmine (M.W. 5000) or 10 μM albumin in 0.05 M potassium phosphate buffer + 1 mM EDTA + 0.1% Tween-80; pH 7.0, at 25°. The concentrations of inhibitor were: –I (●); 10 μM albumin (x); 2.5 μM salmine (□); 7.5 μM salmine (△); and 25 μM salmine (○). (C) Poly-*l*-lysine (M.W. 3000). Cytochrome oxidase (9.3×10^{-9} M heme *a*) was added to a cuvette containing cytochrome *c* and poly-*l*-lysine (M.W. = 3000; PL-3) in 0.05 M potassium phosphate buffer + 1 mM EDTA + 0.1% Tween-80; pH 7.4.

The true K_i must be less than 0.1 nM (see further calculation below). The kinetics are thus "pseudo-non-competitive", and the number of bound polycation molecules is approximately stoichiometric with the available oxidase "molecules". These results are consistent with the findings of Smith and others [2, 9] on the reaction of polycations with mitochondria oxidizing exogenous cytochrome *c*. Table II summarizes the results obtained with Keilin-Hartree particles. Spectrophotometric studies on the inhibition of submitochondrial particles by PL-195 showed that the reaction was relatively slow. Inhibition half times varied from 150 sec with 7 nM PL-195 through 50 sec with 17 nM to 12 sec with 83 nM polycation. The calculated "on" velocity constant is therefore approximately 10^6 M^{-1} sec^{-1}.

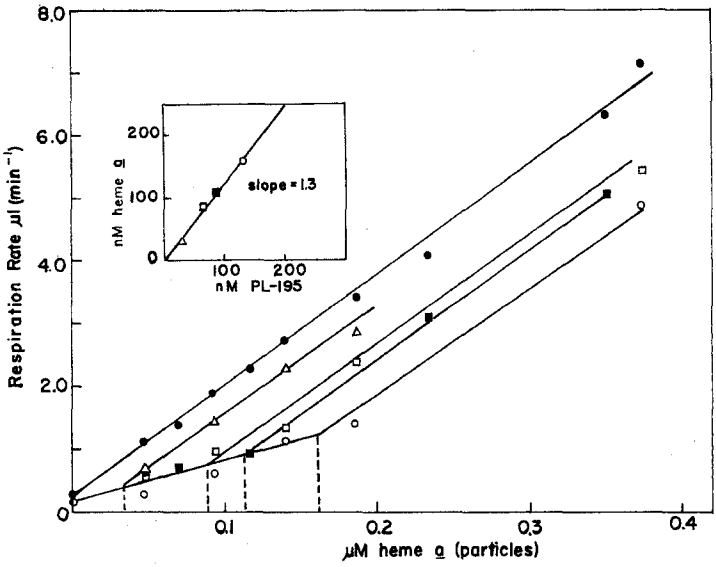


Figure 3. Inhibition of Keilin-Hartree particles by PL-195. Respiration rate (μl O_2 N.T.P. min^{-1}) is plotted against particle concentration for varying amounts of added inhibitor: ○—○, control (no inhibitor); △—△, + 33 nM PL-195; □—□, + 67 nM PL-195; □—□, + 83 nM PL-195; ○—○, + 133 nM PL-195; 6.25 μM cytochrome *c*, 67 mM phosphate pH 7.4, 1 mM EDTA. Manometric experiments, 1.5 ml final volume at 30° C, 33 mM ascorbate, 660 μM TMPD. The *inset* plots the intersections of the various titration curves (in nM heme *a*) against polylysine concentration.

TABLE II. Polycation effects with Keilin-Hartree submitochondrial particles[a]

Polycation	M.W.	Pattern of inhibition	Apparent K_i[b]	[E] /4K_i[c]
Salmine	5,000	Competitive	1-2 μM	0.02
PL-150 and	150,000	Stoichiometric	1-50 nM[d]	≈1.0[e]
PL-195	195,000			
PL-3	3,000	Non-competitive	10-20 μM	0.002

[a] Polarographic experiments: 1 mM TMPD, 10 mM ascorbate; 60 mM potassium phosphate, pH 7.4, 1 mM EDTA, 25°.

[b] Determined from Dixon plots or by titration (cf. Fig. 3).

[c] [E] = oxidase concentration in heme *a* (usually ca. 0.1 μM).

[d] Depends on oxidase concentration (see text).

[e] May increase above this value in "aged" or cholate-treated particles (see text).

Superstoichiometric Binding: Detergent-free Cytochrome Oxidase

Yet a third pattern of inhibition is possible if the solubilized oxidase is assayed under conditions similar to those used with submitochondrial particles and in the absence of dispersing agents such as Tween-80. Salmine, as before, acted as a competitive inhibitor of the oxidase with slightly lower affinity (4-5 μM); PL-3 continued to show non-competitive behavior with a somewhat decreased affinity ($\approx$100 μM). As with the submitochondrial particles, however, PL-150 appeared to bind very tightly, giving 50% inhibition at concentration of $\sim 10^{-10}$ M. The results are summarized in Table III, which may be compared with Table I.

TABLE III. Polycation effects with cytochrome oxidase in absence of detergent (aggregated)

Polycation	M.W.	Pattern of inhibition	Apparent K_i	$[E]/4K_i$ [b]
Salmine	5,000	Competitive	4 μM	6×10^{-3}
PL-150	150,000	Superstoichiometric	0.2 nM	125
PL-3	3,000	Non-competitive	100 μM	2.5×10^{-4}

[a] Direct reading from Dixon plots. The conditions are as described in Table II.
[b] [E] = concentration of cytochrome oxidase (0.1 μM heme *a*).

PL-150 inhibition in this system is "superstoichiometric" in that one molecule of polycation is capable of inhibiting many molecules of oxidase (aa_3). This marked increase in inhibitory capacity of PL-150 is a consequence of the absence of Tween-80. In the polarographic assay, PL-150 had no effect on the rate of cytochrome *c* reduction with either ascorbate or ascorbate plus TMPD. But it inhibited the oxidation of cytochrome *c*, measured either spectrophotometrically or polarographically in the absence of Tween-80, much more than in the presence of detergent. Thus 3 nM PL-150, which inhibited the reaction by only about 70% in the presence of 0.1% Tween-80 (cf. Table I), gave almost complete ($\geqslant$98%) inhibition in its absence.

In this respect Tween-80 is acting as a "liberator" (a substance with small effect in the absence of inhibitor, but markedly increasing turnover in its presence [23]).

The isolation of the oxidase involves breaking up the mitochondria with cholate (*Methods and Materials*). Similarly, treatment of "intact" Keilin-Hartree particles with smaller amounts of cholate to give the "*cholate washed*" particles (*Methods and Materials*) also induces an increased sensitivity to high molecular weight polylysine. Thus, cholate seems to act as a "potentiator" of polylysine inhibition.

Figure 4 illustrates a manometric experiment carried out with cholate-treated particles under conditions similar to those used in Fig. 3. With these particles, 0.25 μM oxidase (heme *a*) was required to titrate 30 nM PL-195 (an aa_3 : PL-195 ratio of 4). In addition, a lag phase now occurs before maximal inhibition is achieved, as observed by Smith and Minnaert [9]. Figure 5 summarizes the results of experiments with cholate-treated particles oxidizing cytochrome *c* under the conditions of the Smith-Conrad assay [1, 2]. When enzyme is added to a PL-195 containing system, or when PL-195 is added to a reaction that is already proceeding, the system slowly becomes inhibited at a rate similar to that described above (10^6 M^{-1} sec^{-1}); this inhibition requires an amount of polylysine approximately equimolar with the heme *a* present. Pre-incubation of the particles with polylysine, however, over a longer period, produced a much greater inhibition in which 0.3 nM PL-195 was able to titrate 3.6 nM oxidase (heme *a*), an aa_3 : PL-195 ratio of 6. The sharpness of the inhibition curve (Fig. 5) indicates that the true K_i is not greater than 0.01 nM.

In contrast to the increased sensitivity of the cholate treated particles, the endogenous *c* of normal Keilin-Hartree particles is not dissociated by polylysine and reactions which depend on the endogenous *c*, including TMPD oxidation, are little affected [5, 9]. The steady state reduction of added cytochrome *c* during succinate oxidation is also unaffected by the presence of polylysine although the rates of reduction on succinate

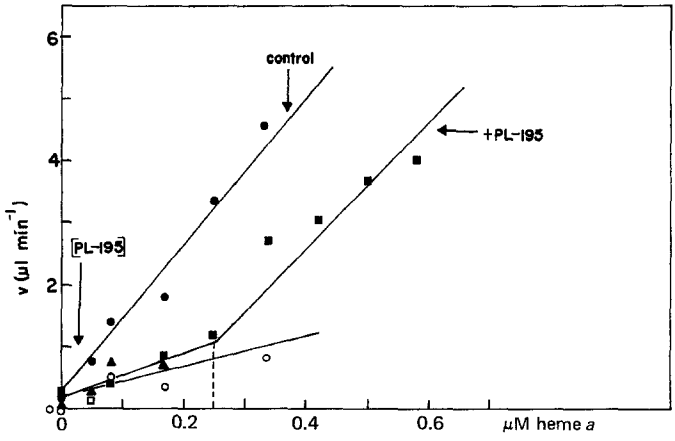


Figure 4. Inhibition of cholate-treated particles by PL-195. Respiration rates are plotted against particle concentration (heme *a*) as in Fig. 3: ●——●, control (+ 12.5 μM cytochrome *c*); ■——■, + 34 nM PL-195 (+ cytochrome *c*); ▲——▲, control (without added *c*); ○——○, + 34 nm PL-195 (without added *c*). 17 mM ascorbate, 330 μM TMPD, 67 mM phosphate, pH 7.4, 1 mM EDTA, manometric experiments at 30° C.

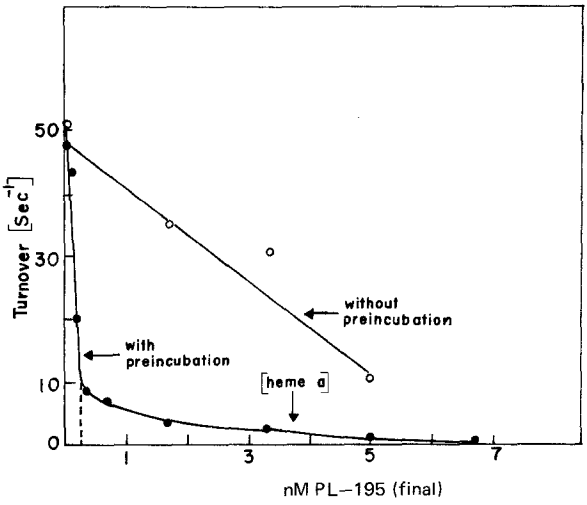


Figure 5. Effect of preincubation on "superstoichiometric" inhibition of cholate-treated particles by PL-195. Oxidation of 30 μM ferrocytochrome *c* followed spectrophotometrically at 550 nm on addition of 3.6 nM (final heme *a* concentration) cholate-treated Keilin-Hartree particles: ●——●, enzyme and inhibitor preincubated together at a concentration ~100x that in final assay for 15-30 min; ○——○ enzyme added to ferrocytochrome *c* solution containing PL-195 at indicated concentration. 67 mM phosphate pH 7.4, 1 mM EDTA, 25° C.

addition and on the transition to anaerobiosis are markedly diminished. In *c*-deficient and cholate-treated particle preparations, addition of polycations increases cytochrome *c* reduction during the steady state.

Previous studies [16] have suggested that the azide sensitivity of the oxidase may be modified by the combination with antibody. Experiments combining the inhibitors PL-150 and azide failed to demonstrate any such interactions. The apparent affinity for azide was independent of the presence of polycation in both the submitochondrial particle ($K_i \approx 120$ μM) and the isolated ("aggregated") oxidase ($K_i \approx 30$ μM). PL-150 also had no effect on the spectroscopic shift of the cytochrome *a* alpha-peak during the azide-inhibited steady state.

Effect of Polycations on the Intact Respiratory Chain in Mitochondria and Submitochondrial Particles

As reported by Smith and Minnaert [9], the rate of respiration with succinate or NADH and the steady state reductions of the cytochromes are not affected by polycations in particles with tightly bound endogenous cytochrome *c*. When cytochrome *c*-deficient (cholate-

treated) particles are used, however, the high molecular weight polylysine inhibits succinate oxidase activity at similar levels to those inhibiting ascorbate-TMPD oxidation. Thus 20 nM PL-195 gave 50% inhibition of cholate particles (0.33 μM in heme *a*, respiring on 60 mM succinate in 67 mM phosphate, pH 7.4) at concentrations of *c* varying from 5 to 35 μM. The apparent K_m for cytochrome *c* was slightly increased (from 10 to 20 μM) by 17 nM PL-195, but at higher PL-195 concentrations the inhibition once again became "pseudo-non-competitive"; the maximum velocity declined from the value obtained in the absence of inhibitor to 10-15% of that value (equivalent to the activity of the endogenous cytochrome *c*).

Although the amount of inhibitor required for complete blockage ($(E)/4\,K_i \approx 4$), and the kinetic behavior at any one inhibitor concentration, closely resemble the results with ascorbate-TMPD, there is some evidence for differences in the response to increasing polycation concentration. Figure 6 shows the inhibition of ascorbate-TMPD oxidase

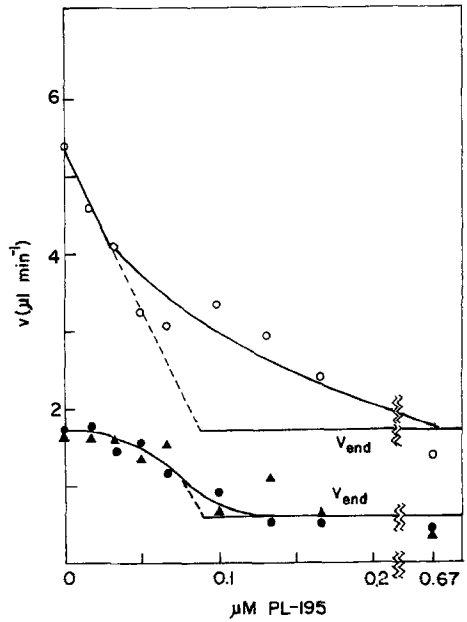


Figure 6. Comparison of ascorbate-TMPD, succinate, and NADH oxidase inhibitions by PL-195. Manometric experiments with cholate-treated particles 0.24 μM heme *a*, 12.5 μM added cytochrome *c*: ○—○, ascorbate (17 mM) + TMPD (330 μM) oxidation; ●—●, succinate (67 mM) oxidation; ▲—▲, NADH (6.7 mM) oxidation. 67 mM phosphate, pH 7.4, 1 mM EDTA, 30° C. Polylysine (M.W. 195,000) added as indicated. "V_{end}" indicates rate of endogenous reaction in absence of added cytochrome *c*, determined separately for the two types of substrate.

activity compared with the inhibition of NADH and succinate oxidase activity at an intermediate cytochrome *c* concentration. The two latter reactions tend to show sigmoid inhibition patterns.

Similarly, the high molecular weight polylysine is able to inhibit the ascorbate-TMPD oxidase activity of cytochrome *c* depleted mitochondria. 300 nM PL-195 gave complete inhibition of this *c*-dependent reaction in depleted rat liver mitochondria (cf. Smith and Minnaert [9]). Although such polycation sensitivity does perhaps provide further support for the idea [14, 18, 24] that the cytochrome *c* binding site is on the outside of the inner mitochondrial membrane, an unusually high sensitivity, like that observed with cholate-treated submitochondrial particles or with the isolated "aggregated" oxidase was not found.

Discussion

Contrary to the report of Takemori *et al.* [3], we find the inhibition of cytochrome oxidase by salmine to be competitive towards cytochrome *c* in both the isolated oxidase and the submitochondrial particle. The non-competitive (or almost "uncompetitive") kinetics observed previously [3] may be attributed to the manometric assay used, in which the reduction of cytochrome *c* by quinol becomes rate limiting at low cytochrome *c* concentration, ensuring that the measured K_m is in fact the sum of the true K_m and a factor proportional to the ratio of enzyme to quinol concentration [25]. Similarly, when reversibility is ensured (as in the presence of Tween-80), the inhibition by high molecular weight polylysines (PL-150 and PL-195) is also competitive in nature. Apparent non-competitive kinetics with these compounds are the result of extremely slow dissociation from tight complexes. With an over-all "on" velocity constant of 10^6 M^{-1} sec^{-1} under our conditions, PL-195 will have an effective "off" constant between 10^{-3} and 10^{-5} sec^{-1} for binding constants between 10^{-9} and 10^{-11} M. Between 10 and 10^3 min incubation with a considerable excess of cytochrome *c* would therefore be required for 50% reversal of inhibition. On the other hand, the low molecular weight PL-3 showed non-competitive kinetics in all three systems studied (dispersed and aggregated oxidase, and Keilin-Hartree particles) at relatively high concentrations. High ionic strength is capable of reducing the maximal oxidase activity [8] as well as increasing K_m for cytochrome *c*. It is possible that polylysine of low molecular weight is binding at a site affecting primarily the rate of electron transfer; the resulting enzyme-polylysine complex may then have a finite albeit slightly reduced affinity for cytochrome *c*, or a second molecule of PL-3 may have to react in order to block the cytochrome *c* binding site. It may be noted that the fully soluble cytochrome *c* peroxidase is much more sensitive than the oxidase to PL-3 ($K_i \approx 0.2$ μM) [26] and that in

this case, despite the general similarity between the peroxidase and oxidase kinetics [27], the inhibition is competitive.

Table IV summarizes the reported inhibitions by various polycations. The varying effectiveness indicates that in addition to the magnitude of the positive charge, the size of the polycation is also important (in contrast to cytochrome *c* peroxidase, where polycation inhibitory effects are almost identical on an equal charge basis [26]). In particular, the ineffectiveness of PL-3 may be due to its failure to meet a "critical size" requirement for competition with cytochrome *c*. Jacobs *et al.* [28] have made a similar suggestion for the polycation-hydroquinone reaction catalyzed by the oxidase.

The stoichiometric and "superstoichiometric" inhibition produced by PL-150 gives a possible way of looking at the location and distribution of membrane binding sites for cytochrome *c* [18]. If one oxidase unit (aa_3) reacts with one molecule of cytochrome *c*, then the total number of binding sites is half the heme *a* concentration (hence the tabulation of (heme *a*)/4 K_i values in Tables I, II and III). The largest number of sites

TABLE IV. Comparison of polycation inhibitions of soluble cytochrome oxidase preparations

Polycation	Oxidase	Apparent K_i value: Molar (M)	Apparent K_i value: Per lysine residue (M)	Reference
PL-3	aggregated[a]	10^{-4}	2×10^{-3}	this paper
	dispersed[b]	5×10^{-5}	10^{-3}	
PL-15	aggregated[c]	10^{-7}	10^{-5}	5
PL-43	dispersed[d]	5×10^{-6}	1.5×10^{-3}	3
PL-150 (or PL-195)	aggregated[a]	$(10^{-9}$ to $10^{-11})$	10^{-6} to 10^{-8}	this paper
	dispersed[b]	7×10^{-8}	7×10^{-5}	
Salmine	aggregated[a]	4×10^{-6}	10^{-4}[e]	this paper
	dispersed[b]	10^{-6}	2×10^{-5}[e]	
Salmine	dispersed[d]	6×10^{-6}	1.5×10^{-4}[e]	3
Salmine	aggregated[c]	10^{-6}	2×10^{-5}[e]	1

[a] Polarographic assay in absence of Tween-80.
[b] Spectrophotometric assay in presence of Tween-80 (0.1%).
[c] Spectrophotometric assay (pH 7, $\mu = 0.11$, no detergent added).
[d] Manometric assay (90 mM phosphate pH 7.4, 20 mM hydroquinone, 1% Emasol 1130).
[e] Per arginine residue.

blocked by one polylysine molecule was about 100 in the case of the solubilized oxidase, which is capable of aggregation onto the long (4000 Å) polymer molecule (Table IV). On the mitochondrial membrane, the oxidase molecules must be on the average 150 to 200 Å apart (cf. the estimate for rat liver mitochondria [29]). As these cannot presumably be approximated more closely, the maximum would be about 25, provided they were distributed randomly. In practice the cytochrome aa_3 : PL-150 ratio did not usually exceed 10. And in freshly prepared particles of the Keilin-Hartree type, the value was close to 1.0 (Fig. 3). That is, in such a particle, the anchoring of a polylysine to one cytochrome *c*-binding site precluded the binding of any other part of the molecule to another site (as if each site lay in its own crevice). Further studies on the inhibition of the cytochrome *c*-deficient mitochondria are needed (cf. Racker [30]) to find out whether there are any conditions under which polycation sensitivity increases, indicating that more than one binding site is occupied by a single polycation molecule. Figure 7 schematically summarizes our present picture of polycation binding.

One assumption in Fig. 7 is that the very tight binding of PL-150 and PL-195 may involve a second site in addition to that occupied by cytochrome *c*, and that this site (but not the cytochrome *c* site) is protected by combination with Tween-80.

The apparent "superstoichiometric" binding behavior of the higher M.W. polylysines introduces some curious kinetic possibilities, some of which may be reflected in actual catalytic responses. For example, the tight binding systems often show a residual (10-20%) activity at high polylysine concentration. A reaction in which a high concentration of cytochrome *c* was capable of displacing a bound polycation from one of a number of binding sites, without causing its release into solution, would

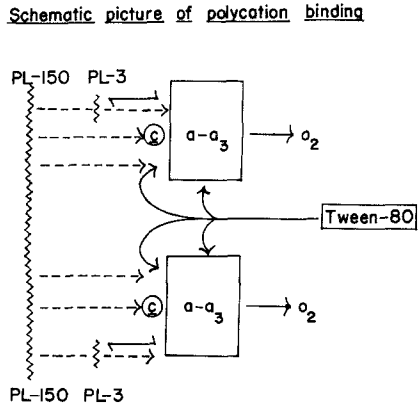


Figure 7.

create such residual activity. Likewise, such a partially dissociated complex would be fully effective in the succinate or NADH oxidase systems where, because the oxidase is present in functional excess, only 10% of sites need be occupied for full activity [14]. Hence the possibility arises of sigmoid inhibition curves, as seen in Fig. 6.

Further uses of the high molecular weight polycations to probe the arrangement and topology [24, 30] of binding sites on the inner mitochondrial membrane may be expected.

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References

1. L. Smith and H. Conrad, *Fed. Proc.*, 17 (1958) 313.
2. L. Smith and H. Conrad, in: *Haematin Enzymes*, J. E. Falk, R. Lemberg and R. K. Morton (eds.), Pergamon Press, Oxford, England, 1961, p. 260.
3. S. Takemori, K. Wada, K. Ando, M. Hosokawa, I. Sekuzu and K. Okunuki, *J. Biochem. (Tokyo)* 52 (1962) 28.
4. P. Person and A. S. Fine, *Arch. Biochem. Biophys.*, 94 (1961) 392.
5. H. C. Davies, L. Smith and A. R. Wasserman, *Biochim. Biophys. Acta*, 85 (1964) 238.
6. W. W. Wainio, *J. Biol. Chem.*, 192 (1951) 349.
7. T. Yonetani, *J. Biol. Chem.*, 236 (1961) 1680.
8. P. Nicholls, *Arch. Biochem. Biophys.*, 106 (1964) 25.
9. L. Smith and K. Minnaert, *Biochim. Biophys. Acta*, 105 (1965) 1.
10. E. Margoliash and A. Schejter, *Adv. in Prot.*, 21 (1966) 113.
11. E. Stotz, J. Connelly and M. Morrison, *Biochim. Biophys. Acta*, 32 (1959) 543.
12. E. Stotz, A. M. Altschul and T. R. Hogness, *J. Biol. Chem.*, 124 (1938) 745.
13. T. E. King, M. Kuboyama and S. Takemori, in: *Oxidases and Related Redox Systems*, T. E. King, A. S. Mason and M. Morrison (eds.), Vol. II, John Wiley and Sons, Inc., N.Y., 1965, p. 707.
14. P. Nicholls, E. Mochan and H. K. Kimelberg, *FEBS Letters*, 3 (1969) 242.
15. B. S. Mochan, R. W. Lang and W. B. Elliott, *Biochim. Biophys. Acta*, 216 (1970) 96.
16. B. S. Mochan, R. W. Lang and W. B. Elliott, *Biochim. Biophys. Acta*, 216 (1970) 106.
17. E. Mochan and B. S. Kabel (Mochan), *Fed. Proc.*, 28 (1968) 880.
18. P. Nicholls, H. K. Kimelberg, E. Mochan, B. Mochan and W. B. Elliott, in: *Probes of Structure and Function of Macromolecules and Membranes*, B. Chance, C. P. Lee, J. Blasie (eds.), Vol. I, Academic Press, N.Y., 1971, p. 431.
19. B. Chance and B. Hagihara, *Proc. 5th Int. Congr. Biochem. (Moscow, 1961)*, 25 (v) (1963) 3.
20. L. Smith and H. Conrad, *Arch. Biochem. Biophys.*, 63 (1956) 403.
21. H. Lineweaver and D. J. Burk, *J. Am. Chem. Soc.*, 56 (1934) 658.

22. M. Dixon, *Biochem. J.*, 55 (1953) 170.
23. T. Keleti, *J. Theoret. Biol.*, 16 (1967) 337.
24. A. N. Malviya, B. Parsa, R. E. Yodaiken and W. B. Elliott, *Biochim. Biophys. Acta*, 162 (1968) 195.
25. T. Yonetani, *J. Biol. Chem.*, 237 (1962) 80.
26. E. Mochan, *Biochim. Biophys. Acta*, 216 (1970) 80.
27. P. Nichols and E. Mochan, *Biochem. J.*, 121 (1971) 55.
28. E. E. Jacobs, E. C. Andrews, H. Wohlrab and W. Cunningham, in: *Structure and Function of Cytochromes*, K. Okunuki, M. D. Kamen and I. Sekuzu (eds.), University of Tokyo Press, 1968, p. 114.
29. M. Klingenberg, in: *Biological Oxidations*, T. P. Singer, (ed.), John Wiley, New York, 1968, p. 3.
30. E. Racker, in: *Essays in Biochemistry*, P. N. Campbell and F. Dickens (eds.), Vol. 6, Academic Press, N.Y., 1971, p. 1.