THE INHIBITION OF CYTOCHROME OXIDASE BY LYSOSOMAL CATIONIC PROTEINS OF RABBIT POLYMORPHONUCLEAR LEUKOCYTES*

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Summary: At 0.33 μM, a mixture of cationic proteins from PMN lysosomes decreased the velocity constant of rat liver cytochrome oxidase by 50%. Separation of the proteins into fractions of circa 8000 and 4000 M.W. reveals that the former is the more potent in its inhibition of cytochrome oxidase , causing 50% inhibition of activity at 0.1 μM.

Polycationic substances profoundly affect mitochondrial respiration (1) and cytochrome oxidase activity (2,3). The lysosomes of rabbit polymorphonuclear leukocytes (PMN) contain six arginine-rich cationic proteins (LCP) which have been implicated in activities of PMN. Such proteins are bactericidal, being inimical to bacterial respiration and membrane integrity (4,5). Since PMN can also be toxic to host tissue cells and membranes and thereby cause increased capillary permeability, edema and localized hemorrhage (5-7), we deem it important to determine whether their LCP might be implicated. Using liver mitochondria as a representative system, we found previously (8) that the LCP could profoundly inhibit mitochondrial respiration: herein we report a possible basis for this activity.

Methods

The acid extraction of cationic proteins from PMN lysosomes has been described (4). The proteins were freed of small contaminants by permeation

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chromatography through Sephadex G-25 and the mixture so derived was used or it was further processed with Sephadex G-50 to yield two fractions, of approximately 8000 and 4000 M.W. (8K and 4K, respectively), each containing three components. Cationic proteins were suspended in 1 mM acetate buffer (pH 5) for use; proteins were determined by the Lowry procedure (9). Cytochrome oxidase was extracted from rat liver mitochondria by use of Tritons X-114 and X-100 (10); such extracts were dialyzed versus 100 volumes of 0.1 M potassium phosphate, pH 7.0:0.1% Emasol 4130 for 18 hours at 4°. Cytochrome oxidase activity was determined at 25° in a CAry model 14 spectrophotometer upon the addition of cytochrome c (95+% reduced) (11). Cationic proteins were preincubated 1 minute with cytochrome oxidase prior to addition of cytochrome c. Cytochrome oxidase concentrations were calculated from Soret band absorbancies of difference spectra

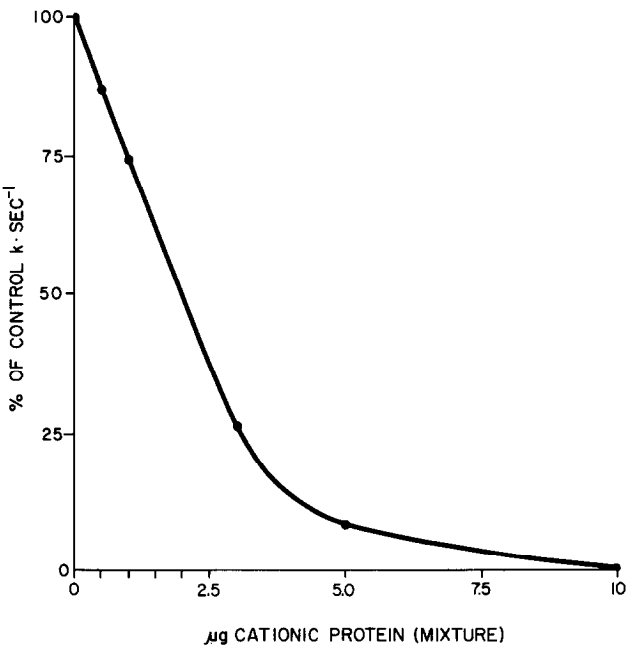

Figure 1. The Effects of Unfractionated Cationic Proteins of PMN Lysosomes on Rat Liver Cytochrome Oxidase. Conditions: 45 µmoles Tris, pH 7.0; 12.2 nmoles cytochrome c; 9.5 pmoles cytochrome oxidase (control $k \cdot sec^{-1} \cdot 10^3 = 5$); plus indicated amounts of cationic proteins in volume of 1.0 ml.

(11,12); first order velocity constants and specific activities were calculated by established procedures (11).

Results and Discussion

In our earlier studies the oxidation of NAD-linked substrates and succinate by mitochondria were equally affected by the LCP mixture or its subfractions (8). In light of this and the work of others (1-3), we began this study by determining the effects of LCP on cytochrome oxidase. A typical experiment is presented in Figure 1. The velocity constant of the oxidase was decreased 92% by 5 µg of LCP; while 50% inhibition occurred in the presence of 2 µg of LCP. On the basis of an average formula weight of 6000, 2 µg/ml equals 0.33 µM. Figure 2 presents the relative activities of the 8K and 4K fractions. When separated on the basis of size, the inhibitory capacity was consistently found to reside predominantly

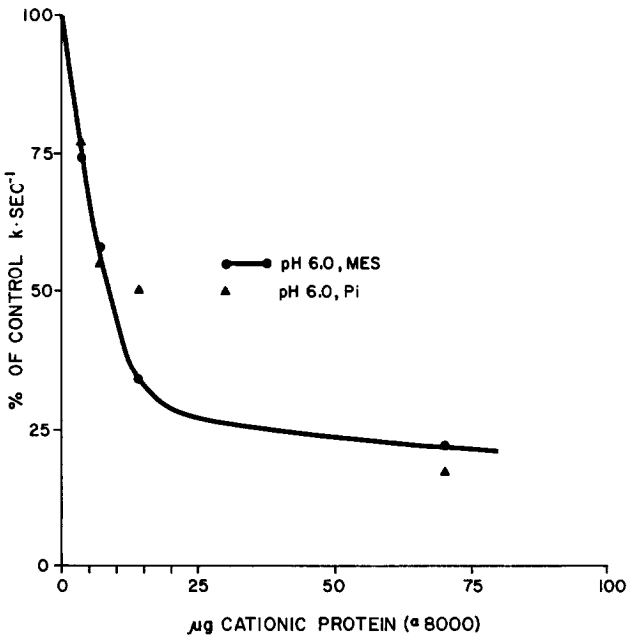

Figure 2. The Inhibition of Rat Liver Cytochrome Oxidase by Subfractions of the Cationic Protein Mixture of PMN Lysosomes. Conditions: 32 µmoles Tris plus 3 µmoles EDTA, pH 7.1; 9.9 nmoles cytochrome c; 2.5 pmoles cytochrome oxidase (control $k \cdot sec^{-1} \cdot 10^3 = 14.3$); 1 ml volume.

in the 8K fraction. In this experiment, the amount of 8K protein needed for 50% inhibition of cytochrome oxidase was one-tenth of the amount of 4K protein needed for a similar effect (Figure 2).

When the effect of varying pH was studied, it was found that the ability of 8K fraction to inhibit cytochrome oxidase is greatly diminished at pH values below neutrality. In the experiment of Figure 3 there was a five-fold increase in the amount of 8K fraction required for 50% inhibition at pH 6.0 compared to pH 7.1 (Figure 2). Essentially identical results were obtained

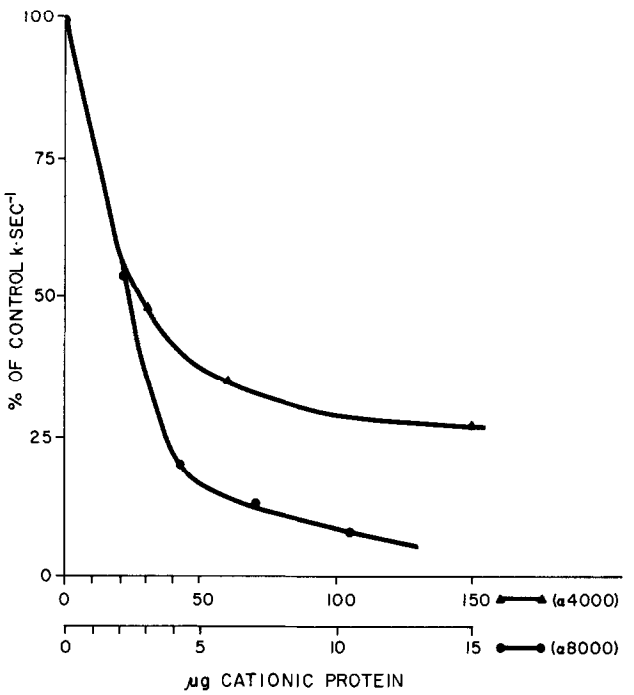

Figure 3. The Influence of pH on the Ability of the 8K Fraction to Inhibit Rat Liver Cytochrome Oxidase. Conditions: 190 μmoles MES and 0.5 μmole EDTA, pH 6.0; 1 pmole cytochrome oxidase (control $k \cdot sec^{-1} \cdot 10^3 = 12.1$). Points plotted with triangles were obtained at pH 6.0 with 23 μmoles potassium phosphate and 0.5 μmoles EDTA as buffer system and with 0.5 pmole cytochrome oxidase (control $k \cdot sec^{-1} \cdot 10^3 = 9.2$). In both cases, 9.9 nmoles cytochrome c and final volume of 1.0 ml.

(Figure 3) under conditions wherein orthophosphate was used as the buffer rather than MES. It is of interest that these characteristics are the direct antithesis of those found for the polycation, protamine. Person and Fine found (2) that the inhibitory action of protamine on cytochrome oxidase was little affected by pH variation over as much as 2 units in the vicinity of neutrality, but that phosphate was strongly inhibitory to protamine action.

The experiment wherein the most potent action of the 8K fraction has been observed to date is presented in Table 1 (in presence of 19.7 nmoles of cytochrome c). Complete inhibition of oxidase activity occurred with 350 pmoles of 8K protein and, when the results were graphed, it was calculated that 50% inhibition occurs in the presence of 100 pmoles of 8K protein - i.e., at a concentration of 0.1 μM. Table 1 presents as well an experiment wherein the effects of varying ferrocytochrome concentrations on the inhibitory action of the 8K

Table 1

The Influence of Cytochrome c Concentration on the Ability of the 8K Fraction to Inhibit Cytochrome Oxidase

Conditions: 50 μmoles Tris, pH 7.4; ferrocytochrome c as indicated; 14.2 pmoles cytochrome oxidase in 1 ml total volume. Figures in parentheses are per cent of control values.

Cationic Proteins	nmoles cytochrome c					
	4.9		9.9		19.7	
pmoles	$k \cdot 10^3$	S.A.	$k \cdot 10^3$	S.A.	$k \cdot 10^3$	S.A.
-	444	154	246	170	102	142
88					54 (53%)	75 (53%)
175	84 (19%)	29 (19%)	102 (42%)	61 (36%)	30 (29%)	42 (30%)
350			0	0	0	0

fraction were determined. At each of three cytochrome c concentrations (4.9 to 19.7 nmoles) which yielded a relatively constant specific activity of cytochrome oxidase, 195 pmoles of 8K fraction gave inhibitions of from 60 to 80%. It should be emphasized that the inhibitions by the 8K fraction at each substrate concentration were identical when calculated in terms of either first order velocity constants or specific activities and that the latter term embodies an expression of substrate concentration.

These experiments determine that the lysosomal cationic proteins have a pronounced ability to inhibit cytochrome oxidase. Fifty percent inhibitions of the first order velocity constant were obtained at 7 and 0.1 μM concentrations of the 4K (Figure 2) and 8K (Table 1) fractions, respectively. Thus, the 8K fraction is 70 fold more active on a molar basis. A similar pattern of activities was found previously in the actions of these fractions on respiration by uncoupled mitochondria (8). On the other hand, with coupled mitochondria only the LCP mixture could completely inhibit coupled respiration; neither the 8K nor the 4K fraction was able to completely inhibit respiration although the 4K fraction gave a greater degree of inhibition than the 8K fraction (8). Thus, the LCP are most effective as the mixture in disrupting the respiratory activity of intact mitochondria, but, with either uncoupled mitochondria or cytochrome oxidase per se, then the 8K fraction is clearly the factor most inhibitory to respiration.

At present our results do not define the mechanism of inhibition of cytochrome oxidase by the 8K fraction. However, we can conclude that the 8K fraction is both more potent than and unlike other polycations in its action. Davies et.al. (3) found poly-L-lysine to be one of the more potent competitive polycationic inhibitors of cytochrome oxidase, giving a 50% inhibition with a cytochrome c/poly-L-lysine ratio of 30. The analogous ratio for the activity of the 8K fraction (Table 1) is 197 - indicating that the 8K fraction is far more active than poly-L-lysine. The dissimilarities in the properties of the 8K fraction and protamine insofar as inhibition of cytochrome oxidase is con-

cerned were mentioned above. It should be added that, while the span of ferrocytochrome c concentrations in the experiment of Table 1 was not great and could not be expanded because of the inherent properties of cytochrome oxidase, the results obtained are not consistent with a competitive inhibition of the oxidase by the 8K fraction. This view is reinforced by the following preliminary observation. While the 8K fraction is transparent to incident light of 400 to 700 nm, its addition to a solution of cytochrome oxidase (reduced or oxidized) causes extensive light scattering throughout the spectrum**. However, this action is not accompanied by changes in the wavelengths or essential magnitudes of the α and Soret bands of the difference spectrum of the oxidase. Although it could not be detected by eye, this phenomenon is consistent with a precipitation process and we intend to explore this possibility in future experimentation. Difficulties inherent in obtaining large quantities of LCP have restricted the scope and range of the experiments undertaken to date. We hope to proceed shortly in elucidating the mechanism of action of the 8K fraction on cytochrome oxidase as well as to determine whether the LCP affect additional aspects of electron transport by mitochondria, and whether the LCP can influence the integrity and function of other membrane structures of animal and bacterial cells.

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