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CALCIUM-RELATED PROPERTIES OF HORSERADISH PEROXIDASE

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SUMMARY

Horseradish peroxidase has been shown to be a metalloprotein in which calcium contributes to the structural stability of the protein. Isoenzyme C and A contain 2.0 and 1.4 moles calcium/mole enzyme, respectively, which can be removed by treatment with guanidine hydrochloride and EDTA. Calcium-free isoenzyme C, but not isoenzyme A, reconstitutes upon addition of calcium and regains enzymatic activity. Free calcium readily exchanges with isoenzyme C, but only to a small extent with isoenzyme A. In addition the role of calcium in maintaining molecular conformation is evidenced by the effects of calcium removal from the isoenzyme C on the thermal stability of the protein.

Retrograde axonal transport of horseradish peroxidase has been a useful tool in investigating the morphology of the nervous system (1). Peroxidase injected near nerve endings is incorporated by the neurons and transported retrogradely to the soma. Neurons of the visual system exhibited selectivity among various peroxidase isoenzymes in that isoenzyme C, but not isoenzyme A is transported (2). During an investigation of the chemical structures responsible for this specificity it was found that horseradish peroxidase contains bound calcium. This communication deals with some of the calcium-related properties of the A and C isoenzymes.

METHODS

Purification of horseradish peroxidase was essentially by the procedure of (3). The C isoenzyme was carried through the first CM-52 chromatography followed by preparative isoelectric focusing (LKB Model 8101). It was usually only necessary to chromatograph the A isoenzyme over a single DE-52 column. Purity was monitored by analytical isoelectric focusing according to the Biorad technical bulletin No. 1030 using a pH gradient of 3-10. Enzymatic activity was measured as described previously (2).

In all experiments dealing with the calcium-related properties of horseradish peroxidase, precleaned dialysis tubing, plastic labware, and distilled -

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deionized water were utilized. Samples of enzyme were passed over a small column of Amberlite MB-3 resin before performing calcium analyses. Calcium concentrations were determined with an atomic absorbtion spectrophotometer or with ^{45}Ca.

Removal of the bound calcium from the isoenzymes was achieved by exposing the peroxidase to 6 M guanidine hydrochloride - 10 mM EDTA, pH 7-8 for approximately 4 hours at room temperature. The solution was then dialyzed overnight in 5 mM EDTA, pH 7.0 followed by extensive dialysis in water.

Reconstitution with calcium was carried out by mixing the calcium free protein with an approximately 10-fold excess of calcium at room temperature for one hour. Excess free calcium was then removed with the Amberlite MB-3 column.

Calcium exchange was determined by incubating the protein with a 4-fold excess of [^{45}Ca] Cl_2 (2.5×10^8 dpm per m mole) for 2-6 hours. Excess calcium was then removed through an Amberlite MB-3 column prior to scintillation counting to measure the protein bound radioactivity. Specific activity of the ^{45}Ca was corrected for calcium contained in the peroxidase.

Equilibrium dialysis was performed on the calcium-free isoenzyme C and the data analyzed by a Scatchard plot (4).

RESULTS AND DISCUSSION

Calcium analyses of native horseradish peroxidase show that isoenzymes C and A contain 2.0 and 1.4 moles calcium per mole peroxidase, respectively (Table I). This bound calcium may be removed by incubation of the peroxidase with guanidine hydrochloride and EDTA. Urea (8 M) could not replace guanidine as a denaturant and the presence of EDTA or EGTA alone removed the calcium only very slowly.

A considerable difference between isoenzymes A and C became apparent when calcium-free peroxidase was reconstituted with calcium. The results in Table I show that while isoenzyme C reconstitutes nearly completely, isoenyme A does not demonstrate any recombination. The differences in calcium reuptake are also reflected in the changes seen in the specific enzymatic activity of the two isoenzymes. The specific activity of isoenzyme C falls to approximately 40 percent after calcium removal, and upon reconstitution with calcium returns to 70% of the initial enzymatic activity. In contrast, the specific activity of isoenzyme A falls to 15% of the initial activity after calcium removal and remains unchanged during incubation of the calcium-free enzyme with excess calcium.

Calcium exchange, which occurs upon incubation of the native protein with

Table 1

Calcium content and specific activity of peroxidase

Treatment	Calcium content (moles Ca^{++}/mole peroxidase) isoenzyme C	isoenzyme A	Specific Activity (Units/mg) isoenzyme C	isoenzyme A
Untreated	2.0 ± 0.13 (5)	1.4 ± 0.19 (5)	3373 ± 916 (100%) (4)	1053 ± 70 (100%) (3)
Guanidine HCl and EDTA	0.03 ± 0.06 (4)	0.11 ± 0.10 (3)	1356 ± 231 (40%) (4)	155 ± 10 (15%) (3)
Reconstitution with Ca^{++}	1.7 ± 0.33 (4)	0.13 ± 0.07 (3)	2349 ± 475 (70%) (4)	167 ± 27 (16%) (3)
Exchange with $^{45}Ca^{++}$	1.7 ± 0.12 (3)	0.28 ± 0.14 (6)		

Calcium removal, exchange and reconstitution are as described in METHODS. Values are mean ± S.D., (n) = number of determinations.

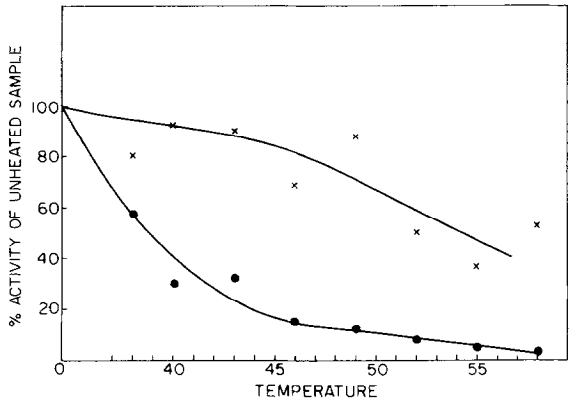

Fig. 1. Thermal Stability of Calcium-free and Native Isoenzyme C. Samples were incubated for 30 min at the indicated temperatures, diluted and assayed immediately. Results are expressed as percent of control sample maintained at 4°. Native enzyme (x), Ca^{++} - free enzyme (●).

^{45}Ca, represents another difference in the properties of peroxidase isoenzymes A and C. Isoenzyme C essentially exchanges completely with free calcium whereas isoenzyme A shows only limited exchange under identical conditions.

A binding constant of isoenzyme C for calcium as determined by equilibrium dialysis was $K_a = 1.6 \times 10^4 \pm 0.44$ (n = 3). The Scatchard analysis also indicated that 1.7 ± 0.15 moles of calcium are bound per mole of isoenzyme C, in close agreement with the calcium content obtained by the nonequilibrium methods. An association constant could not be measured for isoenzyme A since the calcium-free protein did not recombine with calcium.

Since the function of calcium in a number of metalloproteins is to stabilize the enzyme structure (5) the temperature stability of calcium-containing and calcium-free peroxidase isoenzyme C was compared. Figure 1 shows that in the range of 37° to approximately 45° the calcium containing isoenzyme is essentially stable to heat denaturation for a 30 min incubation. In contrast, the calcium-free isoenzyme rapidly loses enzymatic activity and by 45° retains less than 20% of the original activity. At higher temperatures (up to 55°) the calcium containing form of the isoenzyme begins to lose enzymatic activity, eventually falling to approximately 50% of the original level. At this point, however, the calcium-free enzyme is some 10 times lower in specific activity.

The effects of calcium removal on decreasing specific activity and thermal stability strongly suggest that this metal ion functions in maintaining the protein conformation. Experiments are in progress to more clearly elucidate the relationship of calcium to the protein structure.

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