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Correlation of Binding of Rabbit Granulocyte Peptides to *Candida albicans* with Candidacidal Activity†

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NP-1, a candidacidal peptide purified from rabbit granulocytes, bound extensively and with biphasic kinetics to *Candida albicans*. The primary phase of binding was temperature independent and occurred even at 0°C. This primary binding was relatively specific, reversible, saturable, and of high capacity. It was inhibited by increased salt concentrations in the incubation medium, but was relatively unaffected by increasing the calcium ion concentration or by lowering the incubation temperature to 0°C. The secondary phase of binding was only noted under conditions that supported candidacidal activity. Secondary binding was inhibited by millimolar concentrations of calcium, but not magnesium, ions and did not occur at 0°C or when subtoxic concentrations of NP-1 were tested. NP-2 and NP-3a, other potent candidacidal peptides from rabbit granulocytes, also bound directly and extensively to *C. albicans* and competed for binding with NP-1. NP-4 and NP-5, less candidacidally active homologs of the aforementioned peptides, showed relatively little direct binding activity and competed poorly for binding with NP-1 or NP-2. NP-3b, another less candidacidal homolog, bound extensively to *C. albicans*, but did not compete effectively with NP-1 or NP-2. By comparing candidacidal and binding activity of the peptides, we conclude that the candidacidal activity of NP-1 involves primary binding to *C. albicans* followed by postbinding events that are temperature dependent and inhibitable by calcium ions.

Rabbit granulocytes contain six cysteine-rich cationic peptides that express various degrees of antibacterial (3), antifungal (1, 2) and antiviral (R. I. Lehrer, T. Ganz, M. Sherman, and M. E. Selsted, *in* E. Pick, ed., *Lymphokines*, in press) activity in vitro. In the accompanying report, we compared the efficacy of these peptides against *Candida albicans* and identified several factors that inhibited the candidacidal activity of NP-1, the most potent of the peptides (2). These modulating factors included incubation conditions marked by high ionic strength, millimolar concentrations of Ca^{2+}, but not Mg^{2+}, or low temperature (0°C). In this manuscript we compare the ability of the six granulocyte peptides to bind to *C. albicans* with their intrinsic candidacidal efficacy, and we examine the effects of the aforementioned modulating factors on the binding of NP-1 to this organism.

MATERIALS AND METHODS

Peptide purification. The peptides were purified and named as described in the accompanying paper (2).

C. albicans. *C. albicans* strain 820 was cultured in Sabourand 2% dextrose broth for 4, 18, or 66 h as previously described (2). After the blastoconidia were centrifuged (1,000 × *g*, 10 min) and washed twice with 10 mM sodium phosphate buffer (pH 7.4), their concentration was determined by hemacytometer counts and adjusted appropriately. When indicated, heat-killed organisms were prepared by heating viable organisms from 18-h cultures, twice washed with distilled water, in a boiling water bath for 30 min. After three additional washes in distilled water, the heat-killed blastoconidia were counted in a hemacytometer and suspended in the desired buffer.

Binding experiments. Viable or heat-killed *C. albicans* cells, typically 5×10^6 blastoconidia in a final volume of 1.0 ml, were exposed to the indicated NP peptide that had been trace labeled with ^{125}I as described below. Incubations were performed in triplicate in 1.5-ml conical polypropylene centrifuge tubes (West Coast Scientific, Berkeley, Calif.) that contained 0.4 ml of silicone oil (Versilube F50; General Electric Co., Waterford, N.Y.). After incubation for 1 to 60 min at 0°C, room temperature (22 to 24°C), or 37°C, the tubes were centrifuged for 90 s at approximately 12,000 × *g* in an Eppendorf model 3200 microcentrifuge (Brinkmann Instruments Inc., Westbury, N.Y.). Separation of yeast cells from supernatant was complete within 20 s. After the supernatant and silicone oil layers were removed, the tip of the tube was amputated with a razor blade, and the radioactivity of the blastoconidial pellet was counted in a Tracor model 1191 analytic counter (TM Analytic, Elk Grove Village, Ill.). Reproducibility of sampling was excellent, with a mean coefficient of variation for counts per minute from the triplicate samples of <10%. Experimental findings were verified on 2 to 5 (usually three) separate occasions.

Control experiments wherein [^{14}C]sucrose was used as an extracellular fluid marker confirmed that negligible quantities of supernatant fluid contaminated the blastoconidial pellet by this technique (data not shown). Most binding experiments were performed in 10 mM sodium phosphate buffer at pH 7.4, with supplements as indicated below. The effects of calcium and magnesium ions on binding were tested in 10 mM Tris buffer (pH 7.4).

Iodination of NP-1. Two mg of lyophilized NP-1 was dissolved in 0.5 ml of 0.05 M sodium phosphate (pH 7.0), and 2 mCi of $Na^{125}I$ (1.07 mmol) was added in a volume of 20 μl of 0.1 N NaOH. Two IODO-BEADS (Pierce Chemical Co., Rockford, Ill.) were immediately added, and the mixture was incubated for 45 min at room temperature with occasional stirring. Cold KI was added in fivefold (molar) excess with respect to peptide. After a 10-min incubation at room tem-

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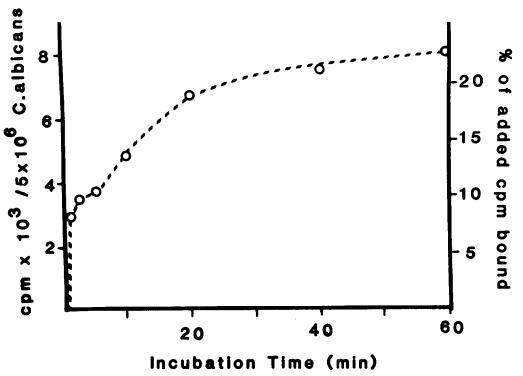

FIG. 1. Binding kinetics of NP-1. *C. albicans* (18 h), 5×10^6 viable blastoconidia per ml, was incubated with 5 μg of NP-1 per ml in 1 ml of 10 mM phosphate buffer (pH 7.4) for up to 60 min at room temperature. The NP-1 contained 0.625 μg of [^{125}I]NP-1 (4.4×10^4 cpm/μg) per ml.

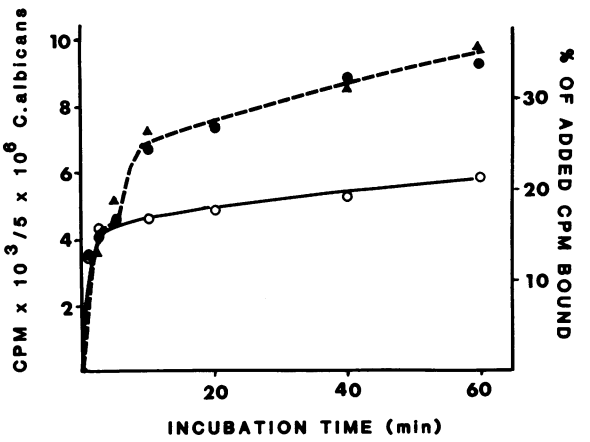

FIG. 2. Effect of temperature on binding of NP-1. *C. albicans* (18 h), 5×10^6 viable blastoconidia per ml, was incubated with 5 mg of NP-1 per ml in 1 ml of 10 mM phosphate buffer (pH 7.4) for up to 60 min at 0°C (○), 23°C (●), or 37°C (▲). The NP-1 contained 0.625 μg of [^{125}I]NP-1 (4.4×10^4 cpm/μg) per ml.

perature, the iodinated peptide was desalted on a 1.0- by 25.0-cm C_4 reversed-phase column (Vydac, The Separations Group, Hesperia, Calif.). Iodinated peptide was eluted at 1.5 ml/min by using as solvents 0.1% trifluoroacetic acid in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). The iodination mixture was injected onto the column, and the elution gradient was developed linearly from 0 to 60% B over 60 min. The iodinated peptide was lyophilized and quantitated by amino acid analysis. Iodinated and native preparations of NP-1 killed *C. albicans* with equal efficacy (data not shown).

RESULTS

Binding kinetics. Figure 1 illustrates a representative binding experiment wherein an 18-h culture of *C. albicans* blastoconidia was exposed to 5 μg of NP-1 per ml, trace radiolabeled with ^{125}I, under incubation conditions that supported effective candidacidal activity (>2 $\log_{10}$ reduction in CFU per milliliter in 20 min). Note that binding was rapid, extensive, and biphasic, with a shoulder between the primary and secondary phase at approximately 2.5 min of incubation. The biphasic nature of binding under these conditions was reproducible when appropriately timed, early samples were taken.

In contrast to results obtained at incubation temperatures permissive for candidacidal activity (23 or 37°C), binding of NP-1 to *C. albicans* differed when experiments were conducted at 0°C (Fig. 2). Although the initial rate of binding was equivalent at all three temperatures, the secondary phase of binding was markedly attenuated at 0°C, a temperature which did not support candidacidal activity.

Binding specificity. We examined the effect of adding excess nonradioactive NP-1 (50 μg/ml) on the binding of ^{125}I-labeled NP-1 to *C. albicans*. The incubation was performed at 0°C to prevent any killing of the test organisms during the incubation. Excess native peptide competed for binding with the radiolabeled material at each concentration tested (Fig. 3). From the molecular weight (3,884) and the specific activity of the NP-1 used in this experiment, we estimated that exposure of *C. albicans* to 5 μg of radiolabeled NP-1 per ml led to the uptake of approximately 2.5×10^7 molecules per blastoconidium and that exposure to 55 μg of NP-1 per ml increased the total binding to approximately 7.2×10^7 molecules of NP-1 per blastoconidium.

We tested the ability of the six native granulocyte peptides to compete with ^{125}I-labeled NP-1 or NP-2 in binding to *C. albicans*. NP-1, NP-2, and NP-3a each competed with radioiodinated NP-1 or NP-2 for binding to *C. albicans* (Fig. 4a). NP-3b was less effective in this regard, and NP-4 or NP-5 had little effect. Excess native NP-1 could rapidly displace radiolabeled NP-1 already bound to *C. albicans* (Fig. 4b). Thus, binding of NP-1 to *C. albicans* exhibited high capacity and saturability (Fig. 3), with relative specificity (Fig. 4a) and reversibility (Fig. 4b).

Other peptides. The binding ability of all six granulocyte peptides to *C. albicans* blastoconidia at 0°C is shown in Fig. 5. Note that even though NP-3b was relatively ineffective in its candidacidal activity, it bound to *C. albicans* as extensively as did NP-1 or NP-2. NP-3a, a peptide with considerable, albeit slow, candidacidal activity in vitro, bound the most extensively of all under these conditions. NP-4 and NP-5, both relatively devoid of candidacidal activity, bound poorly to *C. albicans*.

Modulating factors. Because susceptibility of *C. albicans*

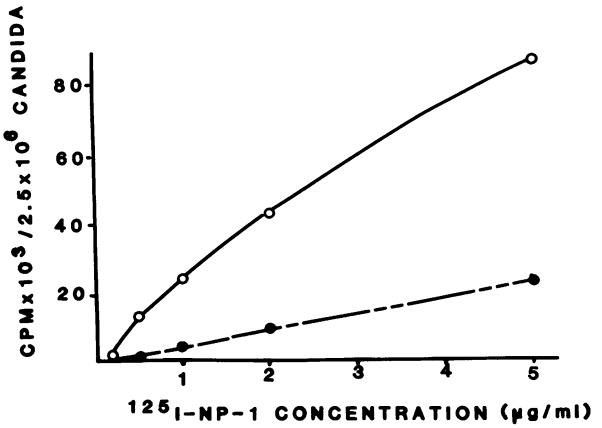

FIG. 3. Competitive binding of native and [^{125}I]NP-1. *C. albicans* (18 h), 2.5×10^6 viable blastoconidia per ml, was incubated for 30 min in a final volume of 0.5 ml with the indicated concentrations of [^{125}I]NP-1 (1.1×10^5 cpm/μg) in the presence (●) or absence (○) of 50 μg of native NP-1 per ml.

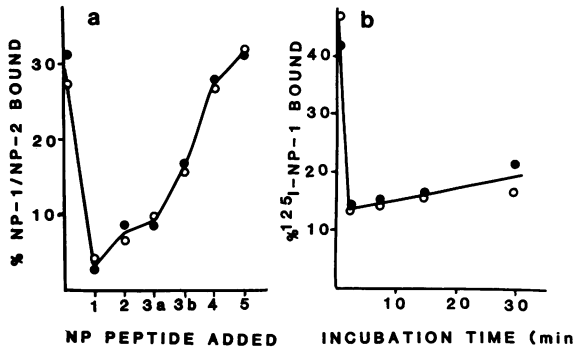

FIG. 4. Specificity and reversibility of NP-1 binding. (a) *C. albicans* (18 h), 5×10^6 viable blastoconidia per ml, was incubated for 30 min at 0°C in 10 mM phosphate buffer (pH 7.4) with 0.5 μg of [^{125}I]NP-1 per ml (●) or 0.5 μg of [^{125}I]NP-2 per ml (○) with or without 50 μg of native NP-1, NP-2, NP-3a, NP-3b, NP-4, or NP-5 per ml. (b) *C. albicans* (18 h), 5×10^6 viable blastoconidia per ml, was initially incubated at 0°C (○) or 24°C (●) with 0.5 μg of [^{125}I]NP-1 (8.4×10^3 cpm/μg) per ml. After 30 min, 50 μg of native NP-1 was added per ml, and the retention of bound [^{125}I]NP-1 was measured. The indicated times refer to minutes after the addition of native NP-1.

to NP-1 varied as a function of culture age, we compared the ability of 4-, 18-, and 66-h blastoconidia to bind NP-1 at 0°C and room temperature (Fig. 6). The susceptible 4- or 18-h cultures showed more binding at 23°C than at 0°C, whereas the resistant 66-h cultures showed equivalent binding at the two temperatures.

Since even modest (25 to 50 mM) concentrations of NaCl markedly decreased the candidacidal efficacy of NP-1, we examined the effects of NaCl supplementation on binding. The addition of 50 mM NaCl decreased binding of NP-1 to *C. albicans* by approximately 82% at 37°C and by approximately 56% at 0 or 23°C (Fig. 7a). Phosphate-buffered saline also rapidly removed approximately 85% of the NP-1 prebound to *C. albicans* under the low-ionic-strength conditions (Fig. 7b). Dilution in low-ionic-strength 10 mM phosphate buffer resulted in slower and less complete removal of bound NP-1, such that approximately 50% dissociated from the blastoconidia after 20 min at either 0 or 23°C (data not shown).

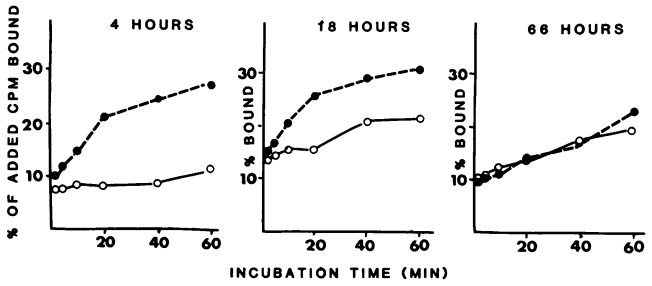

FIG. 6. Effect of culture age on binding of NP-1. *C. albicans* blastoconidia, 5×10^6/ml, were incubated with 5 μg of NP-1 (radiolabeled with 0.63 μg of [^{125}I]NP-1 [3.0×10^4 cpm/μg] per ml) per ml in 10 mM phosphate buffer (pH 7.4) at 0°C (○) or 23 °C (●).

Since low concentrations (e.g., 1 to 2 mM) of calcium, but not magnesium, ions had been found to protect *C. albicans* from the lethal effects of NP-1, we ascertained the effects of these cations on binding. Calcium and magnesium ions had similar, small effects on binding of 0.5 μg of [^{125}I]NP-1 per ml to viable or heat-killed *C. albicans* at 0 or 23°C (data not shown) and on binding of 5 μg of NP-1 per ml to *C. albicans* at 0°C (Fig. 8b). At room temperature, the addition of calcium, but not magnesium, substantially decreased binding of 5 μg of NP-1 per ml to the fungi (Fig. 8a).

We have thus far shown that, whereas binding of NP-1 was biphasic and more extensive under conditions that supported candidacidal activity, it was monophasic and less extensive under nonpermissive conditions. Figure 9 compares binding of NP-1 to viable and heat-killed blastoconidia. Consistent with the previous data, at the permissive temperature of 23°C, viable *C. albicans* cells bound more NP-1 after exposure to 5 μg/ml, a candidacidal concentration, than after exposure to 0.5 μg/ml, a subtoxic concentration. How-

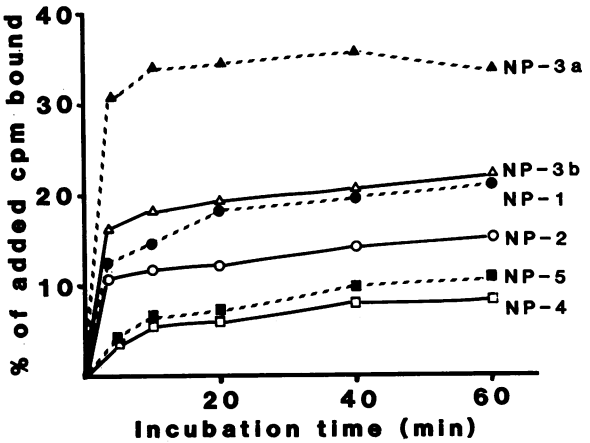

FIG. 5. Comparative binding activity of granulocyte peptides. *C. albicans* (18 h), 5×10^6 viable blastoconidia per ml, was incubated with 5 μg of the indicated peptide per ml in 10 mM phosphate buffer (pH 7.4) at 0°C. All peptides were trace labeled with ^{125}I-labeled homologous peptides. Specific activities and quantities of the tracers: NP-1, 3.9×10^4 cpm/μg, 0.63 μg; NP-2, 9.2×10^3 cpm/μg, 1.25 μg; NP-3a, 7.5×10^5 cpm/μg, 0.42 μg; NP-3b, 5.2×10^4 cpm/μg, 0.63 μg; NP-4, 1.7×10^4 cpm/μg, 1 μg; NP-5, 1.9×10^4 cpm/μg, 1μg.

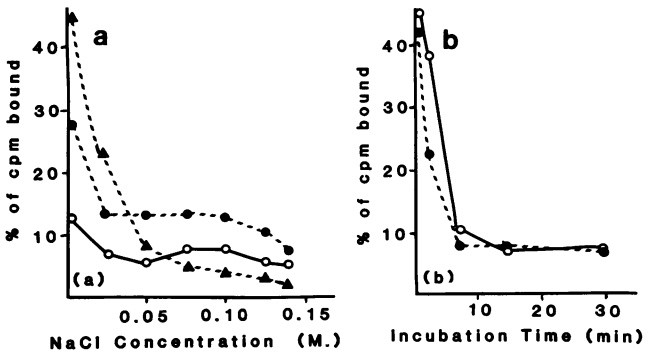

FIG. 7. Effect of NaCl on binding of NP-1. (a) *C. albicans* (18 h), 5×10^6 viable blastoconidia per ml, was incubated with 5 μg of NP-1 per ml for 30 min at 0°C (○), 23°C (●), or 37°C (▲) in 10 mM phosphate buffer (pH 7.4) supplemented with the indicated concentration of NaCl. The NP-1 was trace radiolabeled with 0.5 μg of [^{125}I]NP-1 (1×10^4 cpm/μg) per ml. (b) *C. albicans*, 5×10^6 blastoconidia per ml, was incubated for 30 min at 23°C with 0.5 μg of [^{125}I]NP-1 (8.4×10^3 cpm/μg), centrifuged, and then suspended in 10 mM phosphate buffer supplemented with 0.14 M NaCl. At the indicated times after resuspension, duplicate 100-μg samples were removed and processed by centrifugation through silicone oil.

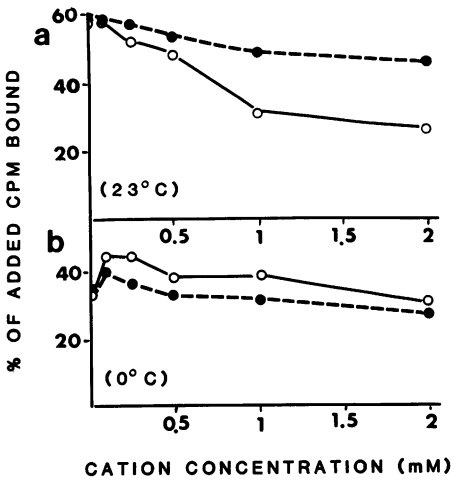

FIG. 8. Effects of calcium and magnesium on binding. *C. albicans* (18 h), 5×10^6 viable blastoconidia per ml, was exposed for 30 min to 5 μg of NP-1 (containing 0.71 μg of [^{125}I]NP-1 [3.8×10^4 cpm/μg] per ml) per ml in 10 mM Tris buffer (pH 7.4) supplemented with calcium (○) or magnesium (●).

ever, heat-killed blastoconidia bound 0.5 and 5 μg/ml to an equal relative extent at 23°C, and heat-killed and viable blastoconidia bound both concentrations of NP-1 equally at the nonpermissive temperature of 0°C.

DISCUSSION

Rabbit granulocytes contain six structurally related peptides that express, to various extents, an impressive range of antimicrobial effects in vitro. Three of these peptides, NP-1, NP-2, and NP-3a, exert potent candidacidal activity in vitro, whereas the other three (NP-3b, NP-4, and NP-5) are substantially less candidacidal. The peptides have been purified to homogeneity, sequenced, and shown to be members of the same gene family (1a).

In this study, we compared the ability of NP-1 through NP-5 to bind to *C. albicans* with their previously established candidacidal potency. As one of the less potent peptides, NP-3b, bound as extensively as did NP-1 or NP-2, binding per se was not highly correlated with candidacidal potency (Fig. 5) effects. The four peptides that bound most effectively each possessed an unusually high net positive charge per molecule (NP-1, +9 net charge; NP-2, NP-3a, or NP-3b, +8 net charge). Their less cationic congeners, NP-4 (+5 net charge) and NP-5 (+3 net charge), bound to a much lower extent. The competitive inhibition data (Fig. 4a) revealed that the more candidacidal peptides (NP-1, NP-2, and NP-3a) competed for common binding sites more avidly than did the less candidacidal ones, NP-3b, NP-4, and NP-5. The relatively poor candidacidal activity of NP-3b, despite its substantial binding to *C. albicans*, suggests that it either binds to noncritical sites or that it is less able to effect the postbinding events required for target cell death.

We studied binding of NP-1 to *C. albicans* in more detail. The biphasic nature of binding curve (Fig. 1) was the initial indication that viable and nonviable *C. albicans* blastoconidia bound NP-1 differently. We had previously noted, based on dye exclusion studies and the release of intracellular ^{86}Rb, that *C. albicans* killed by MCP-1 (NP-1) showed increased permeability (1). Such permeabilization may allow the access of small peptides such as NP-1 to otherwise inaccessible binding sites. Given its polycationic nature, such binding sites for NP-1 would likely include intracellular polyanions such as DNA and RNA. We consider the primary, rapid phase of peptide uptake shown in Fig. 1 and 2 to indicate binding to externally located, accessible sites on nonpermeabilized blastoconidia. Such binding is saturable and reversible and shows relative specificity (Fig. 3 and 4). The secondary phase of binding may reflect uptake by additional binding sites that become accessible to NP-1 after membrane permeabilization occurs. The studies shown in Fig. 9 are consistent with this interpretation.

When candidacidal activity was prevented by conducting the incubation at 0°C rather than at a permissive temperature (23 or 37°C), the primary phase of binding was unaffected (Fig. 2), but the secondary phase was abolished. We conclude that the inhibitory effect of low temperature on peptide-mediated candidacidal activity is mediated via a temperature-sensitive postbinding step, possibly one necessary for permeabilization of the yeast cell.

The abilities of increased ionic strength or calcium ion concentrations to inhibit peptide-mediated candidacidal activity appear to have dissimilar causes. Increasing ionic strength diminished the primary binding of NP-1 to *C. albicans* (Fig. 7a) at permissive and nonpermissive temperatures for both candidacidal (5 μg/ml) and subtoxic (0.5 μg/ml) concentrations of NP-1. We conclude, therefore, that the inhibitory effects of ionic strength arise from the inhibition of primary binding of NP-1 to *C. albicans*. In contrast, calcium ions evidently interfered with peptide-mediated candidacidal activity by acting on some postbinding process. Calcium and magnesium ions had similar effects on primary binding to viable blastoconidia at 0°C (Fig. 8).

Our experiments with *C. albicans* in different phases of growth suggested that the diminished susceptibility of stationary-phase (66-h) blastoconidia to the candidacidal activ-

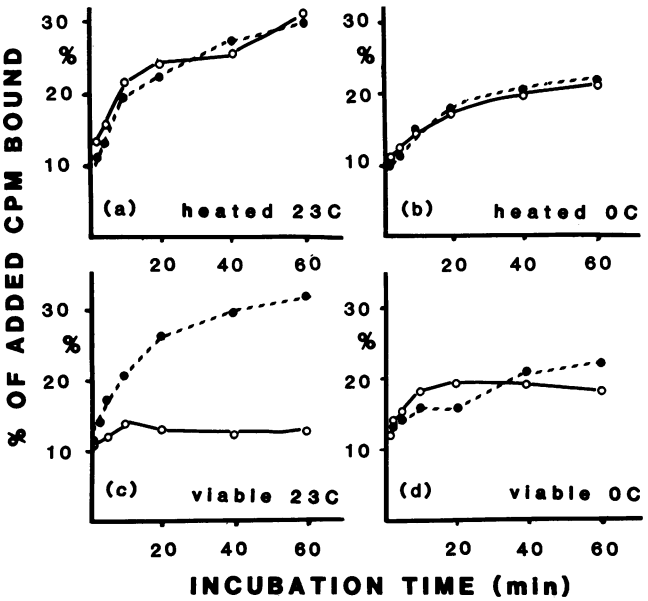

FIG 9. Binding of NP-1 to viable and heat-killed blastoconidia. Viable (c and d) or heat-killed (a and b) *C. albicans* cells were exposed to 0.5 μg (○) or 5 μg (●) of NP-1 per ml, trace labeled with ^{125}I, as in the previous experiments. Incubations were performed at 0°C (b and d) or at 22°C (a and c).

ity of NP-2 was not attributable to diminished primary binding of the peptide relative to that noted with 4- or 18-h-old blastoconidia.

Based on our overall observations, we suggest that the events after exposure of viable *C. albicans* blastoconidia to NP-1 or its active congeners can be divided into four phases: primary binding, postbinding events, permeabilization, and secondary binding. As primary binding is inhibited by increasing ionic strength, it probably occurs electrostatically and is favored by the high net positive charge of the peptides. Postbinding events can be prevented by low temperature or millimolar calcium concentrations and are followed by permeabilization of the blastoconidium. Not only might such permeabilization permit egress of various important intracellular constituents, but also it could permit binding of NP-1 to such potentially important polyanionic molecules as RNA or DNA. Further investigations to elucidate the nature of the postbinding events and to define the primary and secondary binding sites of NP-1 are planned.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. **Patterson-Delafield, J., R. J. Martinez, and R. I. Lehrer.** 1980. Microbicidal cationic proteins in rabbit alveolar macrophages: a potential host-defense mechanism. Infect. Immun. **30**:180–192.

1a. **Selsted, M. E., D. M. Brown, R. J. DeLange, S. S. L. Harwig, and R. I. Lehrer.** 1985. Primary structures of six antimicrobial peptides of rabbit peritoneal neutrophils. J. Biol. Chem. **260**: 4579–4584.

2. **Selsted, M. E., D. Szklarek, T. Ganz, and R. I. Lehrer.** Activity of rabbit leukocyte peptides against *Candida albicans*. Infect. Immun. **49**:202–206.

3. **Selsted, M. E., D. Szklarek, and R. I. Lehrer.** 1984. Purification and antibacterial activity of antimicrobial peptides of rabbit granulocytes. Infect. Immun. **45**:150–154.