Hyperthermia Induced Polyphosphate Changes in *Propionibacterium acnes* as Studied by ^{31}P NMR

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The polyphosphate component in ^{31}P NMR spectra of the Gram-positive *Propionibacterium acnes* increased after hyperthermia treatment. The cells were exposed to temperatures in the interval from 15 °C to 45 °C. The amount of polyphosphate increased with increasing temperature. There were no temperature induced changes in the other phosphorous components seen in the spectra with exception of a decrease in ATP for higher temperatures. The increase in polyphosphates was less than that obtained from cells irradiated by near ultra-violet light.

Introduction

Polyphosphates have been found in several microorganisms [1]. Quite often it is proposed that they simply constitute a high energy source for the cell [1]. But it has also been suggested that polyphosphates can function as so-called "alarmones" in stress situations, as after heat-shocks [2], and trigger defense mechanisms in the cells. Hyperthermia has induced increased amount of polyphosphates both in mammalian [3], yeast [4] and procaryotic cells [2]. The main mechanism might be that the heat treatment induces oxidative stress [5].

In the Gram-positive bacterium *Propionibacterium acnes (P. acnes)* polyphosphates can be observed by ^{31}P NMR [6]. Exposures to near ultra-violet irradiation have shown to increase the amount of polyphosphate [6]. Treatment with red light in the presence of hematoporphyrin derivative, an exogenous sensitizer frequently used in cancer therapy [7], did also induce increased amount of polyphosphates in *P. acnes* [8]. The sensitivity of microorganisms towards near ultraviolet light or photodynamic treatment is due to absorption in chromophores that generate oxygen radicals or singlet oxygen [9]. The mentioned light treatments of *P. acnes* mean, therefore, a large oxidative stress to the cells [10], in turn causing the increased polyphosphate levels.

It was of interest to investigate whether hyperthermia would induce polyphosphate increases in *P. acnes* in the same way as did near ultra-violet irradiation and photodynamic treatment. ^{31}P NMR measurements were performed to study several phosphorous components in the cells. It was of interest to follow not only the polyphosphate levels in *P. acnes* cells during hyperthermia, but also, *e.g.*, the ATP concentrations. In this way one could check if the ATP level changed during and after the hyperthermia treatment as is known to be the case in protozoa and fungi but not in mammalian cells [3].

Methods

P. acnes were grown semianaerobically on synthetic Eagles medium for 5 days [11] or on a complex bactoagar medium with trypton and yeast extract for 2 days. The cells were harvested immediately before an experiment and dissolved in buffer containing an equal volume of 10 mM Na_2HPO_4/10 mM KH_2PO_4/85 mM NaCl/60 mM Mes/100 mM Pipes, including 10% D_2O. The cell suspension (1.5 ml) was made so dense that the cell pellet constituted 30–40% of the total sample volume as described in [6].

The NMR instrument was a Bruker WM-400 spectrometer operating at 161.98 MHz or in some experiments a Bruker AM 500 operating at 212.5 MHz in the pulsed Fourier mode. D_2O was used for the locking mode and without proton coupling. The cell samples were studied in a 10 mm diameter tube. All chemical shifts were measured with respect to 85% *ortho*-phosphoric acid (0.0 ppm), using a reference capillary with 50 mM methylphosphonate purchased from Sigma (δ = 29.4 ppm) as an external standard. Pulses with a flip angle of 60° (20 μsec) and a repetition rate of 1.0 sec were used. The probe head did not spin throughout the accumulation in order to

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avoid inhomogeneous distribution of the cells. The number of accumulations varied from 360 to 720 (corresponding to 6–12 min recording time).

In the experiments where phosphorous components were followed *during* hyperthermia treatment, the NMR sample was heated in the NMR instrument to the desirable temperature. The sample was kept in the instrument for 30 min before the accumulations were performed. The long time needed to achieve a stable temperature was a disadvantage. To avoid the slow heating procedure most of the experiments were therefore performed by heating the NMR sample tubes with the cells in a temperature controlled water bath. The suspension acquired steady state temperature quite rapidly and was kept in the water bath for 15 min. The tube was then placed in the NMR probe head, kept at 22 °C and accumulations were performed. In these experiments we could follow reactions in the cells *after* hyperthermia. Then the sample was again placed in the water bath, the temperature increased 5 °C and the procedure repeated.

Results

Identification of the different peaks observed in the NMR spectra of *P. acnes* was done as in [6]. The peak at −14.3 ppm could not be identified. Spectra were recorded both from cells grown on Eagles or bactoagar medium as described above. Cells grown on Eagles medium contain more polyphosphate than cells grown on bactoagar medium, as mentioned in [1]. When cells were exposed to temperatures changing successively from 15 °C to 45 °C in steps of 5 °C, an increased amount of detectable polyphosphates could be observed in cells grown on Eagles medium. This is demonstrated in Fig. 1. Cells grown on bactoagar medium did not show the same increase, as was also the case after near UV treatment [6].

Few changes could be observed in the other phosphorous peaks after hyperthermia. There was a small decrease in ATP. As long as ATP was present in the cells at room temperature it could also be observed at 35 °C, but as soon as the temperature was increased the ATP decreased, but ADP was still present. For unknown reasons a pronounced ATP signal could not be observed in all cell suspensions. The NADPH peak, however, stayed constant independent of temperature as has also been reported to be the case after near ultra-violet light treatment [6]. In Fig. 2 the ratio between the integrals of the polyphosphate and the NADPH peaks is plotted as a function of temperature. The ratio reflects the changes in the detectable polyphosphates as a function of hyperthermia. The most obvious concentration changes occurred in the temperature interval from 25 °C and 35 °C. Increasing the temperature above this level, caused a slight reduction in the amount of polyphosphates.

During the temperature increase from 15 °C to 40 °C we can observe an approximately three times increase in polyphosphate. This treatment induces 90% cell death in the suspension (data not shown).

The stability of the phosphorous components at a constant temperature was investigated continuously within the sample in the NMR equipment. The temperature in probe head was changed from 22 °C to 35 °C and 40 °C, respectively. The production of polyphosphates was completed and steady state peak height was achieved within 20 min, which is also the time needed to heat the probe head. No further changes occurred as long as the temperature was constant. This was checked for 90 min. Bringing the cells back to room temperature did not give any changes in the spectra, which means that changes detected were irreversible.

The pure handling or transfer to the temperature bath did not change any details in the spectra.

Results from other stress treatments were also achieved. 10% ethanol treatment for 10 min did not increase the polyphosphate level as observed in the NMR spectra. Cold shock treatment, a temperature decrease from 15 °C to 0 °C for 15 min, gave a slight increase in the polyphosphate level.

Discussion

Hyperthermia treatment of *P. acnes* influenced mainly the polyphosphate component observed by ^{31}P NMR. It increased with increasing temperature in the physiological range tested.

The fact that not all phosphate compartments within a cell are "visible" in (liquid phase) NMR [12], makes it impossible from ^{31}P NMR experiments alone to deduce the total amount of phosphate in the cells or a possible effect of temperature on the total polyphosphate pool. In the case of *P. acnes*, the bacteria contain granules in which the polyphosphates are rigidly bond and the relaxation time are too short to allow the polyphosphates to be seen by NMR

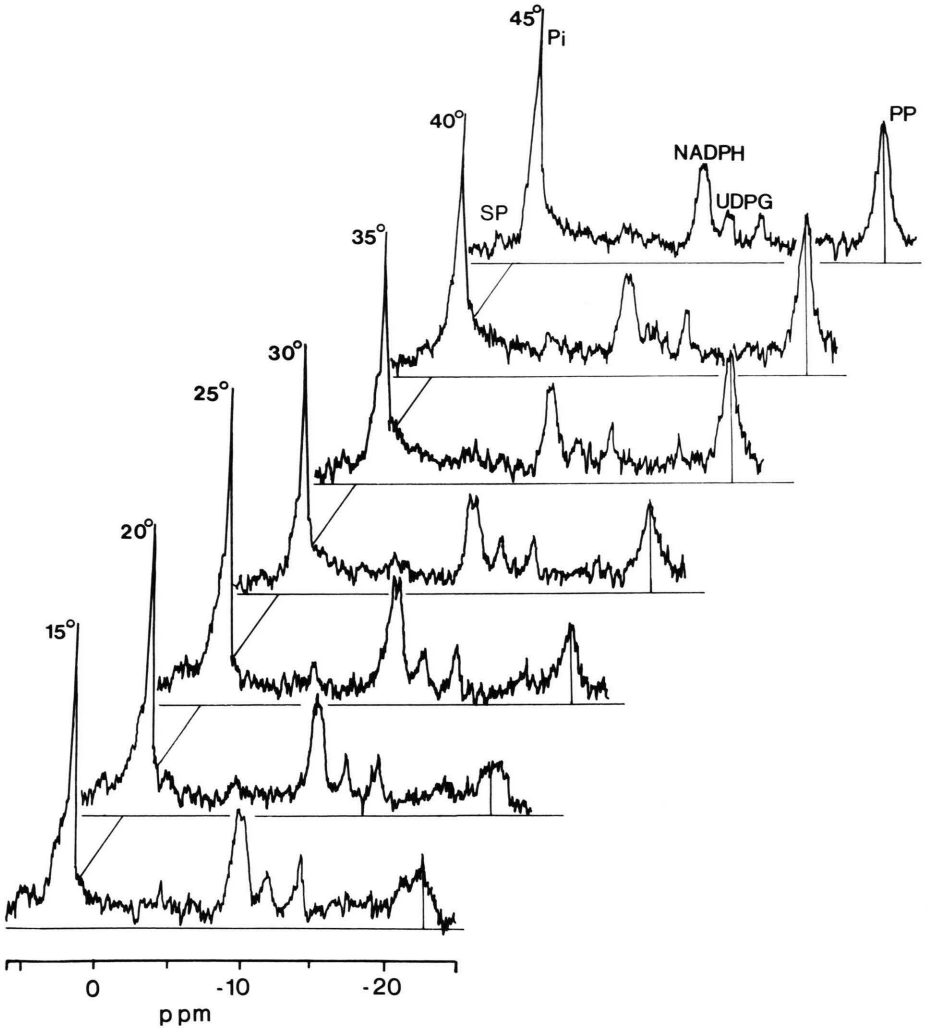

Fig. 1. 161,8 MHz ^{31}P NMR spectra of *P. acnes*, grown on Eagles medium, suspended in buffer with 30–40% cell volume and kept 15 min at different temperatures (indicated). Spectra were recorded at 22 °C immediately after the temperature treatment. Chemical shifts are referred to 85% *ortho*-phosphoric acid. SP, sugar phosphate; P_i, inorganic *ortho*-phosphate; UDPG, uridine diphosphoglucose (right of the two peaks, the left one unidentified); PP, polyphosphates.

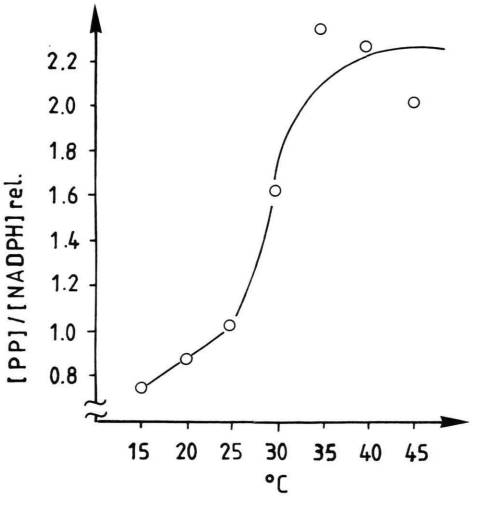

Fig. 2. Ratio between integral values of PP and NADPH peaks as a function of temperature. The curve represent a mean of two complete series of experiments.

(work to be published). For comparison in *Propionibacterium shermanii* at least 70% of the polyphosphates are found as long chain polyphosphates in granules, while the rest is found in other not rigidly bound structures [13]. The changes in the polyphosphate component in *P. acnes* as observed by ^{31}P NMR might, therefore, reflect one part of an equilibrium between long chained "invisible" polyphosphates in granules and short chain "free" polyphosphates in the cytoplasm. Temperature increases might change this equilibrium towards higher concentrations of cytoplasmically dissolved polyphosphates. This would be in accordance with results on *P. shermanii,* where more short length polyphosphates are synthesized at 30 °C than at 20 °C [14]. It is, however, difficult to explain why the increase stabilizes when lethal temperatures of 40–45 °C are reached.

An increase in cytoplasmically dissolved polyphosphates after hyperthermia as found in the present study, might be due to production of tetra- or pentapolyphosphates. Such phosphates have been found in bacteria, fungi and algae after heat shocks [15]. The possibility that they act as so-called "alarmones" for the production of "heat proteins" are discussed in the literature, but the mechanisms have not been elucidated [16]. One explanation might be, as mentioned in the introduction, that hyperthermic treatment induces oxidative "stress" in the cells which in turn increases the amount of polyphosphate [5].

In *P. acnes* the increase in polyphosphates after a hyperthermia treatment which gives 10% survival, is not as large as can be seen after treatment with lethal doses (10% survival) of near ultra-violet light [6] or photodynamic therapy [8]. This shows that there is not a direct relationship between the degree of cell death and the amount of accumulated polyphosphates visible by ^{31}P NMR, as has been reported after cadmium treatment of yeasts [4].

The production of polyphosphates in other cells are not only induced by heat shocks, but also by other stress factors as ethanol treatment [2] or oxidative stress induced by chemicals [5]. We could, however, not find an increased polyphosphate level after ethanol treatment.

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