CONTROL OF OSCILLATIONS OF THE DPNH LEVEL IN A CELL-FREE EXTRACT OF SACCHAROMYCES CARLSBERGENSIS BY 3' 5' CYCLIC AMP

B. Chance and B. Schoener

Johnson Research Foundation,
University of Pennsylvania,
Philadelphia, Pennsylavnia

Received August 27, 1964

Considerable attention has recently been focused upon the nature of cyclic and oscillatory responses of DPNH of reduced pyridine nucleotide in various types of yeast cells (Chance, 1954a; Duysens et al., 1957; Estabrook et al., 1964; Chance et al., 1964a, 1964b; Hommes, 1964), which apparently originate from a glucose activated, cyclic response of cytoplasmic DPNH of yeast cells (Chance, 1954b). More recently (Chance et al., 1964c, 1964d) we have described the general nature of the oscillation in cell-free extracts of S. carlsbergensis (ATTC 4228) and control of the waveform of the oscillations by 5' AMP and by ADP.

In view of the considerable interest in cyclic 3' 5' AMP in regulatory processes in animal tissues and in E. coli as well (Sutherland, 1964), it seems desirable to present a preliminary report on the striking effects of low concentrations of the cyclic nucleotide upon the metabolic regulations involved in the damped sinusoidal oscillations in a cell-free extract of yeast (Chance, et al., 1964c, 1964d). A more detailed report on regulatory phenomena in the cell-free extract will appear shortly (Chance et al., 1964e).

EXPERIMENTAL METHODS

The cell-free extracts are prepared by high pressure extrusion of intact cells as described before (Chance et al., 1964c) and preparations described here are from intact yeast cells stored at 0° for several days. Prior to preparation such cells are aerated for a few hours to remove en-

dogenous substrates and treated with 5 mM glucose just prior to rupturing in 0.1 M phosphate buffer, pH = 6.8. Complete details on the preparation of the method are in press elsewhere (Chance, 1964e). The DPNH level is measured with a double beam spectrophotometer with measuring wavelength set at 340 mμ and reference wavelength set at 400 mμ.

The cell extracts are identified as Type II, and by the number of hours at which the yeast cells have been stored at 0° prior to rupture. For example, in Figure 1 below, the designation of 108 hours refers to the time interval over which the cells were stored at 0° prior to rupture, yet after harvesting.

The 3' 5' AMP was obtained from Sigma Chemical Company and chromatographed as a single peak from Dowex - 1 formate with ammonium formate elution (thanks are due to B. C. Pressman and G. Cheung).

Previous studies (Chance et al., 1964c, d) indicate the usefulness of the oscillating system for demonstrating metabolic control phenomena. The oscillatory system presents two opportunities for testing the response to added substrates and cofactors: as the DPNH level reaches its maximum excursion in the direction of oxidation, it is possible to test the effectiveness of substrates which would accelerate the reduction reaction, and as the DPNH level reaches its maximum level in the direction of reduction, it is possible to test the effects of substances which would activate DPNH oxidation. Inhibitors and activators of DPNH reduction and DPNH oxidation are added at points in the oscillation 180° out of phase. The effects of activators and inhibitors are evaluated in terms of the following parameters : (a) a change in the speed of DPN reduction or DPNH oxidation (b) an alteration in the amplitude or period of the oscillation (c) a change in the steady state DPN/DPNH ratio at which the oscillations take place. Without doubt these are arbitrary criteria but they appear to be the most useful ones in these preliminary excursions into the nature of metabolic control mechanisms in oscillating enzymatic systems.

EXPERIMENTAL RESULTS

Addition of cyclic AMP to the oxidized state. Figure 1 illustrates a typical experimental result on the effect of cyclic AMP on oscillations in the cell-free extract. As described elsewhere (Chance et al., 1964e) the cell-free extract starts its oscillation in the preparative procedure with a 90 minute period at 0°. Upon warming to 25° in the cuvette of the spectrophotometer (1 mm optical path) the period of the oscillations decreases to 12 minutes as shown in the left hand portion of Figure 1.

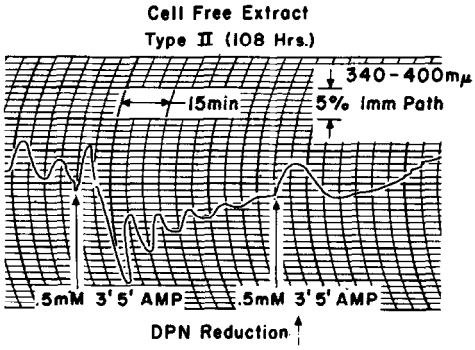

Figure 1. Effect of cyclic AMP on oscillations of cell-free extract of Saccharomyces carlsbergensis: addition to the oxidized state. Recording of the DPNH reduction (upward deflection), recorded at 340 mμ (400 mμ reference wavelength), 1 mm path, absorbancy calibration 5 per cent = 1 large division. Time increases from left to right. Addition of .5 mM cyclic AMP indicated by arrows in the diagram. Preparation II, 69 protein mg/ml, pH 6.8, 25°, .10 M phosphate (Expt. 473 B 9).

At the time of the third maximum of DPNH oxidation (DPNH oxidation is recorded as a downward deflection) .5 mM cyclic AMP is added. This addition, after a 1 minute delay greatly accelerates the reduction of DPN over and above that which would have occurred with normal oscillation; the time for the half maximal reduction after the addition of cyclic AMP is 2.5 minutes. However, the notable feature of the effect of cyclic AMP is not the accelerated DPN reduction but the large oxidation of DPNH which occurs 10 minutes after the addition of cyclic AMP. This remarkable change shifts the whole steady state level of the DPN/DPNH value about which the oscillations occur.

Nevertheless, the period of the oscillations is only slightly shorter (9 minutes). After 4 cycles of oscillation in this mode, the amplitude becomes too small to measure and a second addition of cyclic AMP in this case causes increased DPN reduction with a half time of 3 minutes. Thereafter, only one cycle of oscillation is obtained. While the accelerated DPN reduction caused by both the first and second additions of AMP is in accord with an activation of phosphofructokinase and a consequent activation of DPN reduction at glyceraldehyde-3-phosphate dehydrogenase, the notable effect of the addition is the shift of the steady state level of the oscillating system is in the direction of oxidation. While it is possible that this could be caused by an increased concentration of pyruvate and an increased level of acetaldehyde, the equilibration of alcohol-aldehyde requires less than one minute. With cyclic AMP the steady state level is displaced in the direction of oxidation for more than half an hour.

Addition of cyclic AMP to the reduced state. The fact that the predominant effect of AMP under the conditions of Figure 1 was a shift of the steady state level in the direction of oxidation suggests that a more sensitive response to AMP would be obtained by addition to the maximally reduced state of DPNH.

The response to cyclic AMP at the time of maximal DPN reduction is illustrated by Figure 2.

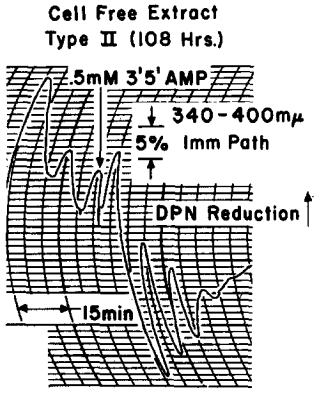

Figure 2. Effect of cyclic AMP on oscillations of cell-free extract; addition to the reduced state. Protein 69 mg/ml. pH 6.8, 25°. Other conditions identical to those of Figure 1 (Expt. 473 B 10).

Here, sinusoidal oscillations of a period of 9.5 minutes are initiated on warming the cell-free extract as in the case of Figure 1. Upon the addition of the same concentration of cyclic AMP as in Figure 1 (0.5 mM) to the third maximum of DPN reduction, there is an immediate jump (less than 0.1 mm) of DPN to the oxidized state as indicated by the abrupt downward deflection (DPNH oxidation is indicated by a downward deflection). After a delay of 1.5 minutes, DPN reduction proceeds to a maximum and thereafter a large oxidation is observed which shifts the steady state level as in Figure 1. The oscillation then proceeds with an increased amplitude and a decreased period (7 minutes) for an interval of approximately 40 minutes.

A comparison of the records of Figures 1 and 2 indicates that the principal effect of cyclic AMP is to cause a large change in the mean oxidation-reduction level about which the oscillations occur in the direction of oxidation together with an increase in the amplitude of the oscillations and an increase of the frequency -- generally, an activation of the oscillatory system. The time between the addition of cyclic AMP and the establishment of the fully oxidized level was approximately 10 minutes in both Figures 1 and 2.

Reactivation of oscillation. Cyclic AMP has the greatest activity of any compound yet tested in the reactivation of extracts in which the oscillations have failed to start or have ceased. This is illustrated in Figure 3.

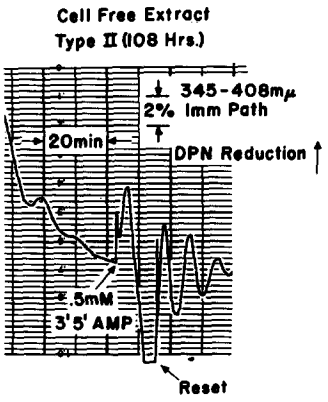

Figure 3. Effect of cyclic AMP on oscillations of cell-free extract: conditions similar to those of Figs. 1 and 2, except that extract is in non-oscillating condition. DPN reduction measured at 345 mμ (408 mμ reference wavelength). Moment of addition of .5 mM cyclic AMP indicated by arrow. At the point marked "reset", the trace was moved two large scale divisions upwards. 69 mg protein/ml; pH 6.8, 25° (Expt. 473 B 2).

The extract showed a rapid change of steady state in the direction of oxidation, with a few damped oscillations superimposed. Upon the addition of 0.5 mM cyclic AMP there is an abrupt reduction of DPN; the half time is 2 minutes. Thereafter, the oscillations start with such a large amplitude that the trace proceeds off scale and is reset upwards approximately two large scale divisions. There follow over three cycles of the oscillation with a period of 8 minutes and a damping factor of 1.15. It is very probable that a lack of cyclic AMP was the reason for the failure of the extract to begin oscillations.

DISCUSSION

Of the various substances which have so far been studied as control factors in the oscillating yeast extract, cyclic AMP acts at the lowest concentrations and has the most striking effects. These effects maintain or enhance the duration of the oscillations, shorten the period, cause a conversion to a more nearly sinusoidal waveform, and shift the average level in the direction of oxidation.

These preliminary experiments clearly demonstrate that the primary response to cyclic AMP of the DPNH level in the oscillating yeast extract is an oxidation. Studies of the effects of a number of substrates indicate oxidants for this system to be acetaldehyde, and its immediate precursors, such as pyruvate and 3-PGA (Chance et al., 1964e). The DPN reductants are glucose-6-phosphate, fructose-1, 6-diphosphate and glyceraldehyde-3-phosphate. The experimental results therefore support the view that in this system cyclic AMP has a primary site of activation between 1, 3-diphosphoglycerate kinase and alcohol dehydrogenase. It will be of interest to isolate the enzyme in this span which responds specifically to cyclic AMP. The data further support the existence of a secondary and less active site for cyclic AMP action which is consistent with the in vitro effects observed upon phosphofructokinase purified from various sources (Mansour and Mansour, 1962; Mansour, 1963; Passonneau and Lowry, 1962). First, the range of concentrations of 3' 5' AMP at which responses are observed with the purified enzyme are

in the range observed in the cell extract (Mansour, 1963). Secondly, a lag in the response of several minutes is observed in both cases (Mansour, 1963). But cyclic AMP cannot produce the observed results exclusively by an activation of phosphofructokinase for the following reasons: 1) from the kinetic point of view, its primary effect is an oxidation of the reduced pyridine nucleotide; 2) from the steady state point of view, the net shift of the DPNH level is in the direction of oxidation illustrated in Figure 1 for approximately half an hour. It seems that other explanations for the overall effect of this compound on the glycolytic system need to be considered. While it is probable that cyclic AMP has generalized effects upon the phosphokinases of the glycolytic system, some unknown condition appropriate for its action exists in the cell-free extracts being studied here; so far cyclic AMP is without measurable effect on kinases isolated from these cells.

SUMMARY

Control of the oscillations of the cell-free system of glycolysis of Saccharomyces carlsbergensis is so far most sensitively affected by cyclic AMP. Its effect upon the oscillations is generally to increase their duration, to convert to a more nearly sinusoidal waveform of a shorter period. The reduced state of the oscillations corresponds more sensitively and more rapidly to cyclic AMP than does the oxidized state, and the general effect of cyclic AMP is to shift the steady state oxidation-reduction level in the direction of oxidation. Cyclic AMP can initiate a previously terminated oscillation in cell-free extracts.

REFERENCES

Chance, B., in The Harvey Lectures, Series XIL,(1954a), p. 145.

Chance, B., in Symposium on the Mechanism of Enzyme Action (W. D. McElroy and B. Glass, eds.) Johns Hopkins, Baltimore,(1954b), p. 453.

Chance, B., Estabrook, R. W., and Ghosh, A., Proc. Nat. Acad. Sci., 51, 1244 (1964a).

Chance, B., Ghosh, A., Maitra, P. K., and Higgins, J. J., N. Y. Acad. Sci., May 26-28, 1964b.

Chance, B., Hess, B., and Betz, A., Biochem. Biophys. Res. Commun., 16, 182 (1964c).

Chance, B., Schoener, B., and Elsaesser, S., Proc. Nat. Acad. Sci., (1964d), in the press.

Chance, B., Schoener, B., and Elsaesser, S., Proc. Nat. Acad. Sci., (1964e), in preparation.

Duysens, L. N. M., and Amesz, J., Biochim. Biophys. Acta, 24, 19 (1957).

Estabrook, R. W., Maitra, P. K., and Chance, B., Symposium on the Mechanism of Cellular Regulation in Bacteria, Marseilles, France, July 1963 (J. Senez, ed.), C.N.R.S., Paris (1964), in the press.

Hommes, F., Biochim. Biophys. Acta, 86, 427 (1964).

Mansour, T. E., and Mansour, J. M., J. Biol. Chem., 237, 629 (1962).

Mansour, T. E., J. Biol. Chem., 238, 2285 (1963).

Passonneau, J. V., and Lowry, O. H., Biochem. Biophys. Res. Commun., 7, 10 (1962)

Sutherland, E., Abstracts, VIth International Congress of Biochemistry, New York, July 1964, S19.