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SYNCHRONOUS REVERSIBLE ALTERATIONS IN ENZYMATIC ACTIVITY (CONFORMATIONAL FLUCTUATIONS) IN ACTOMYOSIN AND CREATINE KINASE PREPARATIONS

SIMON E. SHNOLL and ELIZABETH P. CHETVERIKOVA

Institute of Biological Physics, Academy of Science U.S.S.R., Pushchino and Physical Faculty of Moscow University, Moscow (U.S.S.R.)

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Summary

The phenomenon of synchronism of oscillations of actomyosin and creatine kinase activity in the whole volume of the enzyme preparations was analysed. The synchronous "conformational oscillations" were observed in concentrated gels of actomyosin and in diluted actomyosin and creatine kinase solutions (ATP · creatine *N*-phosphotransferase, EC 2.7.3.2).

The macromolecules of proteins studied may be in two or four conformational states differing in enzymatic activity. Large fluctuations become possible in a range of conditions wherein two or four different states, or conformers, are equiprobable. The synchronization of conformational changes of separate macromolecules is maintained with energy derived, for instance, from some oxidative process or dilution of the solution, the process being displayed as conformational oscillations.

Introduction

In 1958 a phenomenon was discovered which was later called "conformational oscillations" [1,5]. These oscillations appear as sharp reversible alterations of enzymatic activity and of some other properties of proteins. The term "conformational" arose from the fact that these fluctuations, as may be inferred from all the data available, are due to synchronous transitions from one conformational state to another. The macromolecules were shown to exist mainly in two, three or four different discrete conformational states. The intermediate states are less probable. The conformational oscillations, synchronous transitions of macromolecules from one conformational state to another and back, proceed very slowly with a period of oscillation from some seconds to many minutes.

During the past few years this phenomenon has been described by us [1—12,15,21]. In these papers the experimental data are examined in detail

and possible mechanisms of conformational fluctuations are discussed. The synchronization of spontaneous alterations in the whole macrovolume of the protein preparation provides the main evidence of conformational fluctuations. Hence, particular attention was paid to the synchrony of the conformational oscillations in the actomyosin and creatine kinase preparations. The possible mechanisms and physiological importance of the phenomenon observed are also discussed in this paper.

Materials and Methods

Actomyosin (myosin B) was isolated from rabbit muscles by a conventional method [13]. The minced muscles extracted three times by cold water were kept overnight in 0.5 M KCl with 0.03 M $NaHCO_3$. Actomyosin solution was filtered through four layers of cheesecloth and centrifuged at 5000 × *g* for 15 min. The thick gel-like preparation containing 50—60 mg protein per ml was kept at +2° C.

The ATPase activity oscillations were studied in two sets of experiments. In the set "weighed samples", the samples of thick actomyosin preparation were weighed on tracing paper. A pair of weighed samples was put simultaneously into standard test tubes and mixed with 0.45 ml of 0.025 M solution of $NaHCO_3$ which was added simultaneously using a branched double pipette to each test tube. The contents were stirred with two identical Teflon pestles driven by the same motor. Then the tubes were placed into a thermostat and equilibrated at 30° C for 10 min. Then 0.5 ml of ATP (3 mg/ml) buffered with 0.025 ml Tris—HCl pH 8.0 was added with a double pipette to the test tubes. After incubation, the ATPase reaction was stopped with 1 ml 7.5% trichloroacetic acid and the samples were filtered. The sample pairs were taken from the actomyosin preparation at 1- or 3-min intervals and treated identically.

In the second set of experiments ("solutions") the actomyosin preparation was diluted three or five times with 0.5 M KCl, filtered through cheesecloth and equilibrated at 30° C. Two 0.5 ml aliquots of actomyosin solution were taken by a double pipette at 15 or 30 s intervals, placed in test tubes containing 0.5 ml H_2O and stirred by a double-pestled homogeniser. After a 10-min interval, 0.5 ml ATP was added to the samples and they were treated as in the first set ("weight samples"). The phosphate liberated was measured in the trichloroacetic acid filtrates according to [14].

Muscle creatine kinase was purified by a modified Kuby method [15] dissolved in 0.1 M glycine buffer at pH 9.0 (30 mg of protein per ml) and kept frozen. In the ultracentrifuge the preparation was homogeneous and had no myokinase or ATPase contaminants.

The polyacrylamide gel electrophoresis showed two protein bands, a main one and an additional very thin band, both exhibiting creatine kinase activity and locating at the position of the 3 (MM) isoenzyme.

The synchrony of creatine kinase activity oscillations was studied in two kinds of experiments in the following way. In the experiments of the first kind the creatine kinase preparation was thawed and diluted with Tris—acetate buffer pH 7.0 at room temperature to 1 mg/ml concentration. Aliquots of 0.1 ml were taken by a double pipette at 1—3-min intervals and placed in test tubes

containing 9.9 ml of the same buffer. In order to estimate the creatine kinase activity, 0.1 ml aliquots were taken after mixing and placed into test tubes containing 1 ml of substrate mixture at 30° C. The latter contained 3 mM creatine phosphate, 1 mM ADP and 10 mM magnesium acetate in 0.05 M Tris—acetate buffer. The reaction was stopped by *p*-chloromercuribenzoate after 2 min of incubation, and creatine formed was estimated according to Ennor and Rosenberg [16].

In the experiments of the second kind the thawed creatine kinase solution was diluted 5000 times with 0.01 M phosphate buffer, pH 5.2, to $5 \cdot 10^{-6}$ g/ml and placed in a beaker with a magnetic stirrer. Samples of 0.1 ml each were taken from this solution with a double pipette at 30 s intervals for creatine kinase activity estimation by the method mentioned above in 0.01 M phosphate buffer, pH 5.2.

Synchronism of fluctuations in different points of the preparation volume was estimated using the correlation coefficient R:

$$R(x,y) = \frac{\sum_{i=1}^{N} (x_i - \bar{x})(y_i - \bar{y})}{\sum_{i=1}^{N} (x_i - \bar{x})^2 (y_i - \bar{y})^2}$$

where x_i and y_i are enzymatic activity values for two samples in the i-th pair.

$$\bar{x} = \frac{1}{N} \sum_{i=1}^{N} x_i; \quad \bar{y} = \frac{1}{N} \sum_{i=1}^{N} y_i$$

Results

Several examples of ATPase activity fluctuation in weighed samples of actomyosin are shown in Fig. 1. One can see that the values of ATPase activity for different samples of the same protein preparation are different. In most cases the activity difference between two samples simultaneously taken and diluted ten-fold with $NaHCO_3$ is considerably less than between different pairs. Such experiments show that synchronous oscillations of ATPase activity occur in different parts of the gel-like actomyosin preparation and it behaves as a single entity.

The degree of fluctuation synchronism in the experiments presented in Fig. 1 correspond to the following values of correlation coefficient R: 16.02.70, R = 0.57; 14.01.72, R = 0.22; 26.01.72, R = 0.69.

The ATPase activity oscillations in actomyosin solutions prepared by diluting the initial preparation with 0.5 M KCl are shown in Fig. 2. From the figure it follows that the synchronism of oscillation is also observed in the actomyosin solutions.

The degree of fluctuation synchronism in the experiments presented in Fig. 2 is characterized by R values: 16.05.72, R = 0.67; 17.05.72, R = 0.58.

The results of two experiments studying the synchronism of fluctuation in

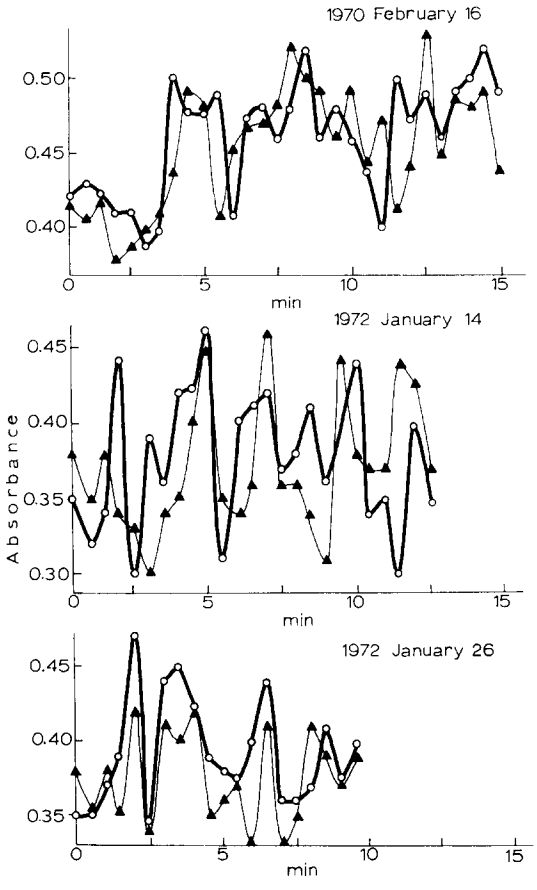

Fig. 1. Illustration of synchronous ATPase activity oscillations in different randomly selected parts of gel-like concentrated (50 mg/ml) actomyosin preparation. ATPase activity was measured in pairs of 50-mg weight samples taken from different parts of the preparation and diluted 10-fold with 0.025 M $NaHCO_3$. The ordinate: ATPase activity in optical density units as a measure of P_i split from ATP; the abscissa: time of sampling, the sample pairs were taken at 30-s intervals.

creatine kinase activity are shown in Fig. 3. A high degree of synchrony of enzymatic activity fluctuations is obvious in the experiments with two enzyme concentrations in the initial solutions, 1 and 0.005 mg/ml.

The degree of fluctuation synchronism in the experiments presented in Fig. 3 is characterized by R values: 11.04.73, R = 0.91; 6.03.74, R = 0.95. It should be mentioned that an intensive stirring of the solution does not alter the synchrony of oscillations.

Usually 4—10 min pass from the moment of preparing diluted solutions of creatine kinase or actomyosin to the beginning of sampling (with a double pipette). In some experiments, we observed a gradual increase in oscillation synchrony in the course of sampling. Such an experiment is shown in Fig. 4, I. The actomyosin preparation in this experiment was the same as that in the experiments shown in Fig. 2. It can be seen that the relative synchrony in this experiment has settled 5 min after the beginning of sampling. For the first 5 min of this experiment, the value of the correlation coefficient R_1 is 0.38,

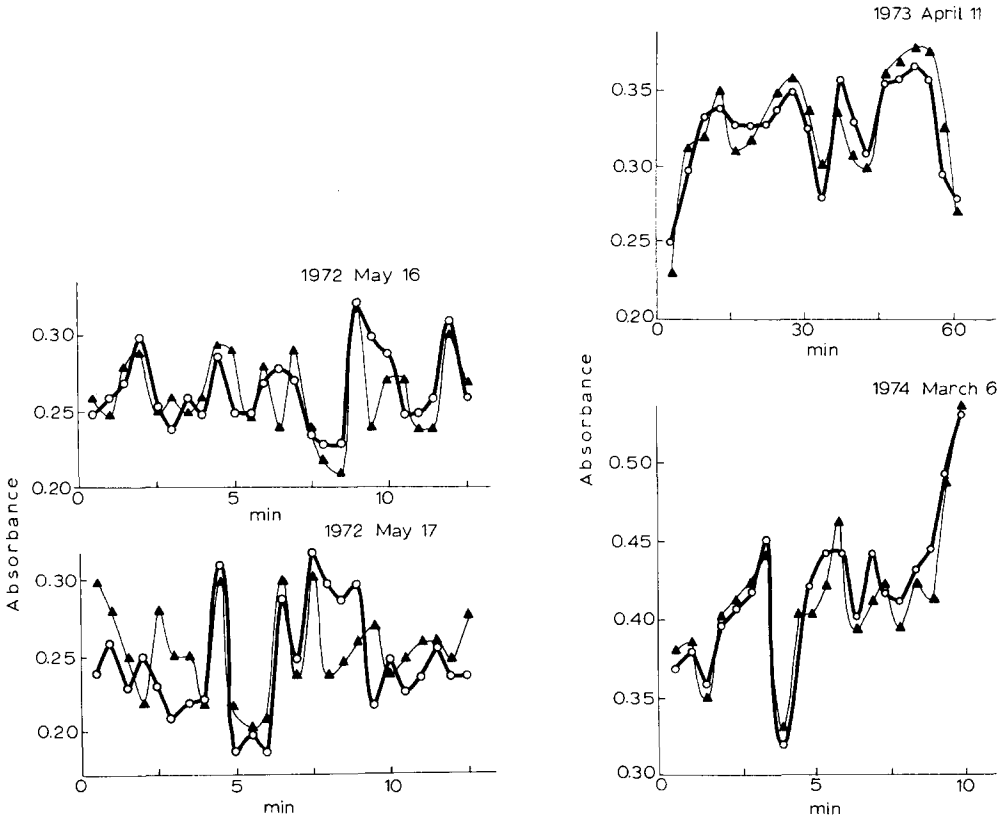

Fig. 2. Illustration of the synchrony of ATPase activity oscillations in the whole volume of actomyosin solution (see text). Designation as in Fig. 1.

Fig. 3. The synchrony of creatine kinase activity oscillations in two randomly selected points of protein solutions. The protein concentration of the initial solution was 1 mg/ml (11.04.73) and 0.005 mg/ml (6.03.74), the time of sampling 3 min and 30 s, respectively. Ordinate, the absorbance of the colored complex of creatine; abscissa, the time of sampling.

for the subsequent 10 min, $-R_2 = 0.86$. As can be seen from Fig. 2, however, synchrony was more frequently observed either at once, beginning with the first sample, or was not observed at all. The experiment with no synchrony is shown in Fig. 4, II. The correlation coefficient for this experiment is $R = 0.04$. Fig. 4, III gives the results of such an experiment with the same actomyosin preparation as in Fig. 4, II but on the next day when oscillation ability disappeared and the average level of ATPase activity decreased nearly two-fold due to absorption of acetone vapour by the preparation. In the context of the present paper the comparison of the experimental results in Fig. 4, II and 4, III shows a sufficiently high accuracy of experimental procedure in all stages. The mean square amplitude in Experiment 4, II amounts to 8% of the arithmetic mean of ATPase activity (0.375 ± 0.032 (8%)) and the mean square amplitude of the standard deviation in 4, III accounts for 1% (0.25 ± 0.002 (0.8%)). The methodical errors are not always as small as in the experiment 4, III and usually range from 2 to 3%.

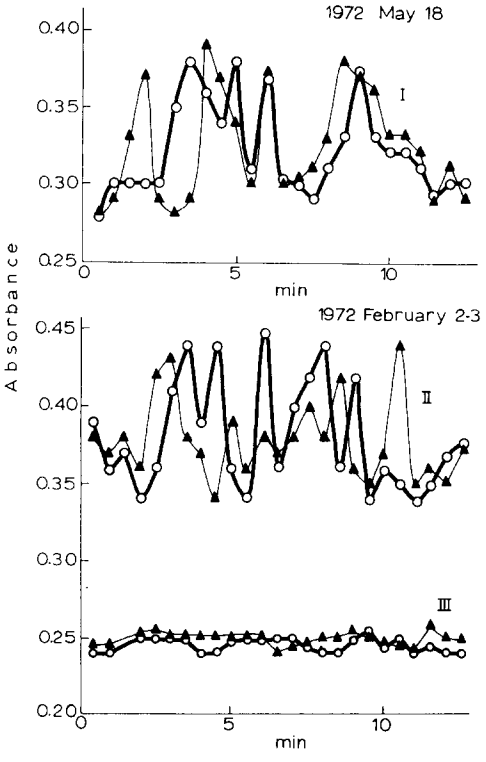

Fig. 4. The examples of experiments, in which the ATPase activity oscillations in actomyosin solutions were synchronized during the course of the experiment (I) or remained not synchronous (II). III, the oscillations were inhibited by acetone. Designations as in Fig. 1, for details see text.

Discussion

As the phenomenon of conformational oscillations was reported and considered by us earlier (1—12), we shall note only the most important aspects.

The data given indicate that the reversible changes in enzymatic activity of actomyosin and creatine kinase occur synchronously at different points of the preparation volume.

It should be noted, however, that estimation of synchronism from R values may be only relative because, for absolute evaluation, the total of the compared periodic or random oscillations must correspond to the normal (Gauss) distribution. At the same time, in the spontaneous fluctuations studied several (usually two, more seldom three or four) discrete states of macromolecules prove to be most probable i.e. the data distribution differs sharply from the normal (Gauss) one.

This problem is discussed in more detail in [1] and [3]. The occurrence of the synchronism of spontaneous fluctuation (mainly that of the number of silver nitrate-titrated SH-groups) in different portions of protein solution was reported by us some 10 years ago [5].

Sharp reversible alterations of the enzymatic activity which are synchronous in the macrovolume of the solution, and some other properties of acto-

myosin and creatine kinase are observed in most, but not all protein preparations obtained. It appears that synchronous oscillations can occur only under combination of many not fully controlled circumstances. Nevertheless, the abundant data accumulated reveal the general features of this phenomenon.

The oscillations consist of transitions of all macromolecules from one conformational state to another, an almost equiprobable one. These transitions stop at the moment when the enzyme molecules interact with their substrates or under the action of other fixators (trichloroacetic acid, *p*-chloromercuribenzoate etc.). The macromolecule "hardens" in the state "catched" to the moment of interaction with the substrate or fixators [1,5].

At present it is impossible to define the time characteristics of "conformational oscillations". We can only note that the preparation transitions from one state to another observed by us occurred no quicker than several times per second. Long-period oscillations are also observed (with an average period of 2—15 min). Revealing the periodical components by methods such as autocorrelation analysis does not provide any sufficiently accurate results because of the relatively small number of the samples in each particular experiment. Therefore, the term "fluctuations" would probably be more exact than "oscillations". However, since the term "conformational oscillations" was suggested many years ago, changing it now is hardly advisable.

Apparently, the energy source for ordered alterations of all macromolecules in the macrovolume is a thermodynamic instability arising from dilution changes in the ionic strength or solution temperature. It was shown earlier [5] that specific redox conditions (a particular relationship between oxidants, reductants and oxygen) are essential for conformational oscillations. Therefore the existence of the oxidative oscillatory reaction seems to be probable. The reactions which may be the model of a periodical kinetics of the oxidative processes have been studied in our laboratory by Zhabotinsky and coworkers in detail [17—18].

A rather strong dependence of conformational oscillation amplitude on very small alterations in the concentration of alcohol, acetone, ethylene glycol etc. was also shown [5,8]. Apparently, these effects indicate the necessity of a specific state of protein macromolecules easily altered upon binding the hydrophobic agents and probably of a specific state of water filling the space between the macromolecules.

The synchronization of alterations in separate molecules in the whole volumes is a necessary condition for revealing the phenomenon of conformational oscillations. Several mechanisms of such synchronization are possible. The oscillating redox reaction in the solution can cause the synchronization of alterations of separate molecules. The state of macromolecules is then only a consequence of alterations in redox conditions in the system and in this case the protein is a passive indicator of a low-molecular chemical oscillatory reaction. The synchronization can be due to the molecule interactions through the surrounding medium. In this case, the oscillations observed reflect the properties of protein macromolecules themselves. The following seems to be possible in the second case. With a specific combination of conditions, two or more different conformational states of macromolecules become equiprobable. More or less large-scale fluctuations, transitions from one state to another, begin.

Each of the conformational forms primes probably the transition of the neighbouring molecules to the same state [5,6,12]. As a result, each fluctuation gives rise to a "wave of structural rearrangement" like the propagation of the crystallization front, as an energy source providing the propagation of the wave can serve some form of thermodynamic instability: slow oxidation of SH-groups, relaxation to a new ionic strength, etc.

It is clear that the conformational oscillations observed depend on both the ability of single macromolecules to transfer from one discrete conformational state to another and the possibility of waves of structural rearrangement spreading.

Independent conformational oscillations at different points of the volume are synchronized by phase entrainment until the whole preparation begins to oscillate as a single entity in conditions favouring the propagation of waves of structural transitions.

We suppose the conformational oscillations to be somewhat like the phenomenon of "critical opalescence" and other manifestations of macroscopic fluctuations under critical conditions. In this case, periodical oscillations are also possible. Some external mechanisms presetting the period or definite properties of the system itself are necessary in order to provide the regular oscillations.

As one can judge from the similarity of the oscillations of muscle proteins studied by us to the phenomenon observed by Duffy [19] on lactate dehydrogenase, in the solution of this protein conformational oscillations also occur. Events like those described by us were observed by Attalah and Lata [20] on serum albumin.

Conformational oscillations of proteins, synchronous in a macrovolume, may be of biological importance. First of all, these oscillations give evidence for the existence of several equiprobable conformational states of macromolecules under some conditions. The transition of macromolecules from one conformational state to another both spontaneous or externally induced, may serve as a mechanism of control of the macromolecule functions. One has the impression that the "oscillatory regime in critical regions" is the most common cause for the periodicity of physiological processes. For example, the actomyosin preparation from skeletal muscles behaves like a heart surviving in vitro. It is possible that the rhythm inherent in the protein is inhibited in skeletal muscle and released during the isolation of actomyosin.

Conformational oscillations are not only evidence of the ability of separate macromolecules to occur in different conformations, but are the result of macromolecule interactions. This makes it possible to pose the question concerning the interaction of acting enzyme molecules resulting in the macroscopic structures. These macromolecular interactions may have a direct relation to the processes of biological morphogenesis [6]. The conformational oscillations synchronized throughout the macroscopic distances may also be the physical ground of biological motility and nerve pulse transmission.

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