PROTEINOID MICROSPHERES AND THE PROCESS OF PREBIOLOGICAL PHOTOPHOSPHORYLATION

M. P. KOLESNIKOV

Bakh Institute of Biochemistry, U.S.S.R. Academy of Sciences, Moscow 117071, U.S.S.R.

Abstract. A chemical model of prebiological photophosphorylation with participation of hemoproteinoid microspheres, mixed microspheres containing bonded riboflavin and microspheres obtained from glycine rich proteinoids was studied. The illumination of aqueous solutions containing microspheres, K_2HPO_4, ADP and electron acceptor leads to an increase of ATP concentration and to a decrease of concentration of inorganic phosphate. Initial photochemical reactions with participations of proteinoid microspheres could have evolved in the course of chemical evolution and led to the emergence of the photophosphorylation in its modern biochemical form.

1. Introduction

According to hypothesis of A. A. Krasnovsky, during the period of chemical (prebiological) evolution an important role was played by process of photochemical activation of substrates (Krasnovsky, 1974). Porphyrins and their complexes with proteinoids have been considered as primary photosensitizers because these compounds are sensitizers in some model reactions of photooxidation and photoreduction (Weber, 1970; Kolesnikov *et al.*, 1979, 1981, 1984; Fox *et al.*, 1978). Proteinoids containing bonded metalloporphyrins (hemoproteinoids) are suitable model compounds for studying of probable ways of prebiological evolution.

The purpose of our work was to study participation of hemoproteinoids, hemoproteinoid microspheres and mixed microspheres containing bonded riboflavin in the reaction of photophosphorylation of ADP to ATP. As shown earlier, illumination of solutions containing proteinoid, hemin dimethylester, AMP and inorganic phosphate (in dimethylacetamide) led to phosphorylation of AMP to ADP (Weber, 1970). Fox and his colaborators have also observed the photosynthetic formation of ADP and ATP in suspensions of proteinoid microspheres (Fox *et al.*, 1978, 1980; Bahn and Fox, 1981), however satisfactory results were obtained only in organic solvents. In the context of chemical evolution on the Earth studies of photophosphorylation reaction in the water medium and at physiological-meaning values of pH are more relevant than these studies in organic solvents.

2. Methods

The following model system have been studied in our experiments:

$$P_i + ADP + A + \frac{\text{proteinoids or}}{\text{microspheres}} \xrightarrow{\text{light}} ATP,$$
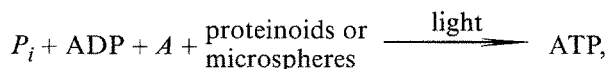
when:

   A – electron acceptors:
       parabenzoquinone or
       riboflavin
   P_i – inorganic phosphate (K_2HPO_4)
   ADP – disodium salt of adenosin-5'-diphosphoric acid, Reanal Co)

To obtain the proteinoids dry mixtures of L-amino acids (Reanal Co) containing 1–3% hemin were heated at sealed evacuated ampules for 4–6 hr at 185–190 °C. Three initial mixtures of amino acids were used in our experiments

(1) 4:4:1:3 (glutamic acid, aspartic acid, lysine and mixture of 9 amino acids in equimolar proportions: histidine, cysteine, glycine, alanine, proline, valine, leucine, isoleucine and phenylalanine).

(2) 12 amino acids in equimolar proportions: (lysine, alanine, glycine, valine, proline, leucine, isoleucine, histidine, cysteine, phenylalanine, aspartic and glutamic acids)

(3) 4:1:1 (lysine, histidine and 10 amino acids in equimolar proportions: cysteine, glycine, alanine, proline, valine, leucine, isoleucine, phenylalanine, glutamic and aspartic acids).

Histidine (or glycine) rich proteinoids were prepared from initial mixtures, containing 25% of these amino acids and 75% of 4:4:1:3 mixtures.

Reaction products were purified by acetonitrile (or methylene chloride) extraction and by subsequent gel filtration on Sephadexes G-25 and G-50 and in model experiment only the material with a molecular weight of over 5000 was used (Kolesnikov et al., 1979, 1984). To hydrolyze of imide linkages resultant proteinoids were treated with NH_4OH solution and evaporated (Fox and Nakashima, 1967).

Microspheres were obtained by the well-known method of Fox (Hsu and Fox, 1976) with small modification. Boiling solutions of hemoproteinoids (in water), containing a few drops of NH_4OH, cooled slowly to room temperature. Suspensions of microparticles were dialyzed against water (Kolesnikov and Maksudova, 1990).

Mixed microspheres were prepared by combination of hot solutions of two proteinoids (Hsu and Fox, 1976).

To obtain microspheres containing bonded riboflavin 100 ml of boiling solution of proteinoid was cooled to 80 °C and then 1.0 ml of warm riboflavin solution (80°, 5×10^{-4} M) was added to this medium and the mixture was allowed to cool slowly to the room temperature. Microparticles obtained were then separated from solution by means of a glass filter (N4) and were washed by water.

Inorganic phosphate in solutions and iron content in hemoproteinoids were measured colorimetrically as described in the our preceding papers (Kolesnikov et al., 1979, 1984). Riboflavin content was estimated by fluorescence analysis.

To obtain the purified preparation of ADP it was twice passed through the DOWEX-1 column, after that the preparation contained only 0.15 – 0.5% ATP as a cotaminant. The experimental solutions were illuminated with incandescence lamp (500 W) through a two-lensed condenser and infrared filter (5% $CuSO_4$

solution). The experiments were carried out in Tunberg tubes at the constant temperature value (tubes were incubated at 19–21 °C in waterflowing vessel during the whole period of illumination). The tubes were illuminated without removal of air at pH value (7.2 –8.0). The periods of illumination were 30, 60, and 90 min. The experimental solutions were made of hemoproteinoids or microspheres suspension (2.0×10^{-6} g Fe per ml, 0.5 – 1.0 ml), K_2HPO_4 (special purity, 0.06M, 0.5 ml), ADP (0.02M, 0.5 ml), parabenzoquinone (5×10^{-4} M; 0.1 ml) or riboflavin (5×10^{-4} M; 0.3 ml) and tris-HCL-buffer (0.05 M, total volume 3.0 ml). Similarly the experimental solutions contained 3×10^{-5} mol K_2HPO_4, 10^{-5} mol ADP and 15–50 nmol ATP (as admixture).

To determine the ATP amount luciferine–luciferase method was used (Strehler and Totter, 1954). The smallest ATP quantity which could be measured under our experimental conditions was 1.5–2.5 nmol.

Microspheres suspension was removed from experimental solutions by filtration (glass filter N 4). The material of five tubes (illuminated under identical conditions) was mixed and this solution was concentrated to minimum volume (0.3–0.5 ml). This solution was then passed through the DOWEX-I column (8×250 mm) eluted with 0.1 M NH_4OH, 0.003 M HCL (AMP), 0.02 M NaCl in 0.01M HCL (ADP) and 0.2M NaCl in 0.01 M HCL (ATP). We used uv-spectral chromatographic detector allowing to measure the optical density of the small portions of eluate (15 microlitres, 260 nm). The last fraction (containing ATP) was evaporated (in vacuum) and then it was dissolved in 1.0 ml of 0.1 M phosphate buffer (pH 7.4) containing $MgSO_4$. The purity of this solution was confirmed by chromatography on Whatman 3MM paper (with standard ATP preparation). Aliquots of this solution were added to the luciferine-luciferase system and the intensity of bioluminescence was measured by chemoluminoscop of special construction (Goncharova and Goldfeld, 1990). The ATP amount was determined by using of calibration curve (standard ATP solutions). The data (Tables I and II) were calculated with respect to one tube. All calculations were carried out by taking into consideration the results of dark and light controls without proteinoids and light controls without K_2HPO_4 (photo-catalyzed dismutation reactions).

3. Results and Discussion

As shown earlier (Sukhorukov et al., 1966) the interaction of orthophosphate and ADP with such electron acceptor as methylene blue, parabenzoquinone or riboflavin led an increase in anionic radicals of acceptor (by EPR-data) and a decrease in orthophosphate (pH of experimental solutions 8.0–11.0).

The present communication gives experimental results concerning influence of light on this system in the presence of hemoproteinoids and proteinoid microspheres. We have discovered that the illumination did not exert noticeable influence on the system ADP – K_2HPO_4 – parabenzoquinone. However, after addition to the solution of hemoproteinoids (or microspheres formed from these proteinoids),

TABLE I

Photophosphorylation with participation of hemoproteinoids, microspheres and riboflavin

Composition of the solutions. The solutions are balanced with respect to iron (1–5) and riboflavin (6–9) content.	ΔATP, nmol ($\pm$ 1.5)	
	In light	In dark
1. P_i + ADP + A (parabenzoquinone)	15.5	12.0
2. P_i + ADP + A + hemin	20.0	12.0
3. P_i + ADP + A + histidine rich hemoproteinoid 4:4:1:3	55.0	15.5
4. P_i + ADP + A + microspheres obtained from histidine rich hemo- proteinoid 4:1:1:3	95.0	15.0
5. P_i + ADP + A + mixed microsperes obtained from histidine rich hemoproteinoid and proteinoid 4:1:1 (in ratio 2:1)	126.0	15.0
6. P_i + ADF + A (riboflavin)	40.0	15.0
7. P_i + ADP + A + histidine rich hemoproteinoid 4:4:1:3	64.0	15.5
8. P_i + ADP + A + microspheres obtained from histidine rich hemo- proteinoid	110.0	10.5
9. P_i + ADP + mixed microspheres: a. riboflavin + histidine rich proteinoid 4:4:1:3	150.6	10.5
b. riboflavin + proteinoid 4:1:1	155.0	12.5
c. riboflavin + proteinoid 4:1:1 + histidine rich proteinoid 4:4:1:3	160.0	12.0

Δ ATP – change in the ATP amount as compared with the control (in the course of 90 min).
The concentrations of the reagents are given in the text.

TABLE II

Photophosphorylation with participation of glycine rich proteinoids and microspheres

Composition of the solutions. The concentrations of reagents are given in the text	ΔATP, nmol ($\pm$ 1.5)	
	In light	In dark
1. P_i + ADP + proteinoid 4:4:1:3	20.5	10.0
2. P_i + ADP + glycine rich proteinoid 4:4:1:3	80.5	10.5
3. P_i + mixed microspheres: a. glycine rich proteinoid + neutral proteinoid obtained from 12 amino acids (in ratio 1:3)	105.6	15.0
b. glycine rich proteinoid + histidine rich proteinoid 4:4:1:3 (in ratio 1:2)	118.4	15.0
c. glycine rich proteinoid + proteinoid 4:1:1 (in ratio 1:2)	144.5	16.0
4. P_i + ADP + glycine heated alone for 6 hr at 190 °C	12.5	10.5

Δ ATP – change in the ATP amount as compared with the control (in the course of 90 min).
The solutions are balanced with respect to fluorescence intensity at 500 nm (that indicates to the flavin-like structures).

illumination accelerated both: the disappearance of orthophosphate and the ac-
cumulation of ATP in solution. ATP synthesis was confirmed by the luciferine-
luciferase assay.

Table I shows the synthesis of ATP under different experimental conditions.
Hemoproteinoids of different composition formed different amounts of ATP. The

neutral hemoproteinoid obtained from 12 amino acids and hemin gave the smallest effect. Acidic hemoproteinoids (4:4:1:3) showed an intermediate effect, and basic hemoproteinoid (4:1:1) showed the largest effect. When the microspheres were used instead of proteinoids, we have observed a further accelerations of ATP accumulation. The mixed microspheres produced the maximum effect on ATP accumulation.

We also found that the consumption of inorganic phosphate in the experimental solutions during the period of illumination exceeded the quantity necessary to account for ATP formation (according to the theoretical calculations). In the special experiment we have established the fact of bonded inorganic phosphate formation (probably pyrophosphate). This fact was established by hydrolysis of dry product of reaction with 1 M HCL (60 min at 100 °C) and following determination of inorganic phosphate. Also a part of initial phosphate was bonded to the proteinoid microparticles and could be liberated only by 0.1 M HCL hydrolysis.

In the system ADP – K_2HPO_4 – riboflavin we found that the illumination caused orthophosphate disappearance with simultaneous synthesis of ATP molecules. The ATP synthesis was also confirmed by the luciferine-luciferase assay. As was reported earlier (Losinova *et al.*, 1983, 1986) the illumination of similar system by visible light resulted in the photosensitized synthesis of ATP. Our data are consistent with this statement and confirm that illumination leads to rise of anionic radical of ADP. The flavo-semiquinone sensitizes this process (according to EPR-data). Presumably, the anionic radical of ADP reacts then with inorganic phosphate forming ATP molecules.

Hemoproteinoids (or microspheres) added to experimental solution appear to accelerate the photophosphorylation reaction (see the Table II).

When the mixed microspheres (containing bonded riboflavin) are used, the phosphorylation reaction is accelerated. As shown in the Table I, mixed microspheres (as compared with riboflavin) accelerate ATP synthesis more than two times.

According to our data, the solution of hemoproteinoid in 0.1 M NaOH (pH 8.0) possess characteristic EPR-spectra when the narrow singlet signal (g ≈ 2.003) disposes on the descending branch of wide line concerning to the iron ions. This singlet signal enlarges its intensity when the experimental solution are irradiated by visible light (400–480 nm) and it returns to initial value after the light is turned out. The light-dependent signal is probably caused by flavin-like system incorporated to polypeptide molecules of proteinoids. This assumption was confirmed by studying of the glycine rich proteinoid. The molecules of glycine rich proteinoid 4:4:1:3 (25% glycine in the initial mixture of amino acids) have the flavin-like structures bonded to the polypeptide chains. The flavin-like character of these structures has been established by meaning of fluorescence emission and fluorescence excitation spectroscopy (see Figure 1). The flavin-like compound was derived from crude product of thermal polycondensation of glycine-enriched amino acids mixture by extraction with organic solvents and then it was purified by column chromatography ($MgCO_3$-supercel) and chromatography in the thin layer (Silufol UV-254 plate).

Heinz and Ried (1981) also reported that flavin-like structures can be formed
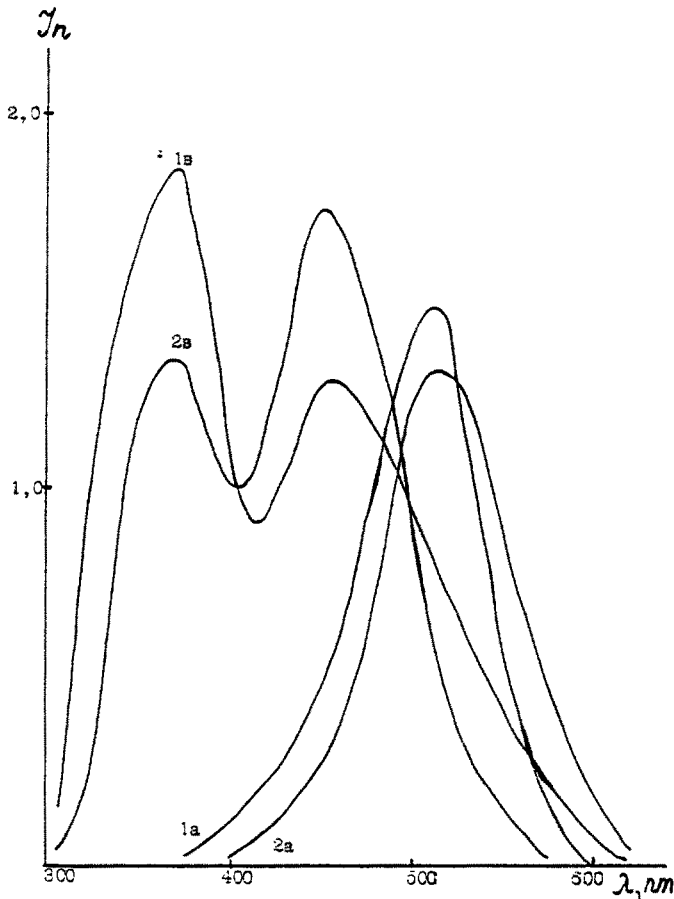
Fig. 1. Fluorescence emission (a) and excitation (B) spectra of the product of thermal polycondensation of glycine-enriched mixture of amino acids (1) and authentic riboflavin (2). The concentrations (in 80% ethanol): 5×10^{-4} M (riboflavin), 0.1 mg/ml (product of thermal polycondensation). Spectrofluorimeter Hitachi MPF 4.

under conditions of thermal polycondensation of dry mixtures of amino acids. Our results are in agreement with this interpretation.

Glycine rich proteinoid microspheres directly sensitized photophosphorylation of ADP to ATP in the presence of inorganic phosphate (Table II). In this case the reaction takes place without adding an electron acceptor to experimental system.

It is known that proteinoids and microspheres catalyze hydrolysis of para-nitrophenyl phosphate and ATP (Hsu and Fox, 1976; Kolesnikov et al., 1979b). However, in the light hemoproteinoids and glycine rich proteinoids apparently cause the competing process – photophosphorylation of ADP to ATP. In this capacity microspheres are superior to the corresponding activity of proteinoids from which they were obtained. This photochemical behavior of the proteinoids and their microspheres gives them an advantage over dark catalysts and suggests that they

can be considered as model of prebiotic photosensitizers.

We do not consider proteinoid photophosphorylation as a model of modern biochemical ATP formation. Initial reactions of the photosynthetic formation of energy-rich phosphates could have been inherited by primitive organisms and led to the emergence of modern mechanisms of ATP synthesis of living cells.

Acknowledgement

References

Bahn, P. R. and Fox, S. W.: 1981, *BioSystems* **14**, 3.

Fox, S. W., Adachi, T., Stilwell, W., Ishima, Y., and Baumann, G.: 1978, *In Light Transducing Membranes*, Academic Press Inc., p. 61.

Fox, S. W., Adachi, T., and Stilwell, W.: 1980, *In Solar Energy*. International progress, Pergamon Press, Vol. 2, p. 1056.

Fox, S. W. and Nakashima, T.: 1967, *Biochim. et Biophys Acta* **140**, 155.

Goncharova, N. V. and Goldfeld, M. G.: 1990, *Origins of Life* **20**, 309.

Heinz, B. and Ried, W.: 1981, *BioSystems* **14**, 33.

Hsu, L. L. and Fox, S. W.: 1976, *BioSystems* **8**, 89.

Kolesnikov, M. P., Voronova, N. I., and Egorov, I. A.: 1979a, *Dokl. Akad. Nauk S.S.S.R.* **248**, 472.

Kolesnikov, M. P., Voronova, N. I., and Egorov, I. A.: 1979b, *Dokl. Akad. Nauk S.S.S.R.* **249**, 1008.

Kolesnikov, M. P., Voronova, N. I., and Egorov, I. A.: 1981, *Origins of Life* **11**, 223.

Kolesnikov, M. P., Maksudova, N. I., and Egorov, I. A.: 1984, *Dokl. Akad. Nauk S.S.S.R.* **275**, 1253.

Kolesnikov, M. P., Maksudova, N. I., and Egorov, I. A.: 1986, *Origins of Life* **16**, 117.

Kolesnikov, M. P. and Maksudova, N. I.: 1990, *Biophisika S.S.S.R.* **35**, 742.

Krasnovsky, A. A.: 1974, in S. Fox *et al.* (eds.), *The Origins of Life and Evolutionary Biochemistry*, Plenum Press, p. 223.

Losinova, T. A., Diomina, O. V., Nedelina, O. S., Brzevskaya, O. N., and Kayushin, L. P.: 1983, *Biophisika S.S.S.R.* **28**, 555.

Losinova, T. A., Nedelina, O.S., and Kayushin, L. P.: 1986, Biophisika S.S.S.R. **31**, 10.

Strehler, B. L. and Totter, J. R.: 1954, in Glick D. (ed.), *Mthods Biochem. Anal. I*, 341, N.Y.

Sukhorukov, B. I., Kirpichnikova, N. P., Blumenfeld, L. A., and Zenin, S. V.: 1966, *Biophisika S.S.S.R.* **11**, 526.

Weber, A. L.: 1970, *Federation Proc.* **29**, 939.