STUDIES ON AGGREGATED MULTIENZYME SYSTEMS: EFFECTS OF BASIC PROTEINS AND PHOSPHOLIPIDS ON OXIDASE REACTION CATALYZED BY FLAVOPROTEIN–IRON–SULFUR–PROTEIN COMPLEXES*

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1. Introduction

Spinach ferredoxin-NADP reductase (FNR) and ferredoxin (Fd) form a tight 1:1 complex under low ionic strength conditions with a dissociation constant of about 10^{-8} M [1–4]. Similarly adrenodoxin reductase (AR) and adrenodoxin (Ad) form a firmly bound 1:1 complex (J.W. Chu and T. Kimura, to be published).

As previously reported [4, 5] cytochrome *c* greatly stimulates the oxidase activity of these complexes and the stimulation is independent of the oxidation–reduction state of the added cytochrome *c* [5]. Since cytochrome *c* is a basic protein, these observations urged us to investigate the effects of biologically important basic proteins, such as histones and protamines, on the oxidase activity of the spinach and adrenal flavoprotein–iron–sulfur–protein systems.

We report in this paper the successful demonstration of stimulation of oxidase activity catalyzed by the flavoprotein–iron–sulfur–protein complexes, which results from interaction of the histone with cardiolipin. Histone stimulates approximately 4-fold the rate of O_2-uptake by ferredoxin-NADP reductase–ferredoxin and adrenodoxin reductase–adrenodoxin systems. Among 12 other proteins and amino acids tested, cytochrome *c* and myoglobin were also effective in stimulating oxidase activity. Cardiolipin further increased the histone-stimulated oxidase activity of the spinach system about 3-fold, however, it completely abolished the oxidase activity of the adrenal system. Other phospholipids tested had no effect on either system. To observe enhanced oxidase activity in the presence of cardiolipin, a specific order for the addition of the reductase–ferredoxin complex and histone was necessary.

2. Materials and methods

FNR and Fd were purified from spinach [6, 7]. AR and Ad were prepared from bovine adrenal glands [8]. NADPH (type II), histone (calf thymus, type II), protamines (salmine, grade I; clupeine, grade III), cytochrome *c* (horse heart, type III), chymotrypsinogen A (bovine pancreas, type II), lysozyme (egg white, grade I), myoglobin (equine skeletal muscle, type I), trypsin inhibitor (soybean, type I-S), bovine albumin (fraction V) and poly-L-lysine (type II) were purchased from Sigma Chemical Co. Phospholipids were obtained from General Biochemicals Co. and dispersed in an ethanol-diethyl ether mixture (1:1, v/v) at a concentration of 1 mg/ml. Reduced cytochrome *c* was prepared as described elsewhere [5]. O_2-uptake was measured with a Gilson KM Oxygraph equipped with a Clark oxygen electrode at 25° in 0.01 M Tris-HCl buffer, pH 7.4.

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3. Results and discussion

The effect of various proteins on the O_2-uptake of the FNR–Fd and AR–Ad systems is presented in table 1. Both systems showed essentially similar behavior toward the specific protein added. Among the proteins tested, cytochrome *c* (both oxidized and reduced forms), histone and myoglobin were effective in stimulating the oxidase activity but other proteins were without effect.

Because Fd and Ad are acidic proteins, it is conceivable that the stimulation is due to an interaction between the iron–sulfur protein and a basic protein, such as cytochrome *c* (pI = 10) or histone (pI = 11). However, some specificity must be required for the stimulation to occur, since protamines (pI = 12) and lysozyme (pI = 11) did not stimulate the activity while myoglobin (pI = 7) was somewhat effective. As an approximation, it appears likely that the content of lysine in the protein but not of arginine is the basis of the specificity for this stimulation (table 1, second column). Free L-lysine and poly-L-lysine are exceptions. As shown in the table; histone could increase the rate of O_2-uptake of both the spinach and adrenal systems about 4-fold under the given conditions. It should be emphasized that the final product of oxygen reduction by the histone-stimulated reaction is mainly H_2O_2, since the molar ratios of added NADPH to total oxygen consumed were calculated to be 1:1 in both the FNR–Fd system and the AR–Ad system. This is in contrast to that found for the cytochrome *c*-stimulated oxidase reaction of the spinach system, where the product has been identified to be H_2O [5].

The effect of phospholipids on the histone-stimulated oxidase activity of the flavoprotein–iron–sulfur–protein systems was then investigated. As shown in table 2, lecithin, phosphatidyl ethanolamine and sphingomyelin had no significant effect on the activity. The acidic phospholipid, cardiolipin, on the other hand, further increased the rate of O_2-uptake of the FNR–Fd system about 3-fold without affecting the total O_2-uptake. Contrary to the spinach system, the histone-stimulation was completely abolished by cardiolipin in the case of the adrenal system. These results clearly demonstrate that cardiolipin behaves differently from other neutral phospholipids, and that its specificity is recognized in different manners by the spinach and adrenal systems. One may possibly ascribe this phenomenon to the difference in the acidity of the iron–sulfur proteins**.

In the presence of cardiolipin, the addition of

Table 1
Effect of proteins on the oxidase activity on the FNR–Fd and AR–Ad systems.

Addition†	[Lys] / [Arg] + [Lys]	Relative activity	
		FNR–Fd system	AR–Ad system
None	–	1.00*	1.00*
Cytochrome *c*	0.88	9.5	4.9
Histones	0.4 ~ 0.95	3.7	4.4
Myoglobin	0.82	3.9	2.2
Chymotrypsinogen A	0.78	1.8	1.4
Bovine albumin	0.71	1.0	0.89
Trypsin inhibitor	0.52	1.0	0.88
Lysozyme	0.35	1.2	0.95
Salmine	0.0	1.2	1.1
Clupeine	0.0	1.1	1.2
L-Lysine	1.0	1.1	1.0
L-Histidine	–	0.98	1.3
L-Arginine	0.0	1.1	0.92
Poly-L-Lysine	1.0	0.83	0.37

† Proteins and amino acid were added at a concentration of 0.06 mg/ml.

* Specific activities of the FNR–Fd system and AR–Ad system were 5.3 and 10.4 moles O_2/min/mole of the flavoprotein, respectively.

Table 2
Effect of phospholipids on the histone-stimulated oxidase activity of the FNR–Fd and AR–Ad systems.

Phospholipid	Specific activity (moles O_2/min/mole enzyme)	
	FNR–Fd system	AR–Ad system
None	19.4	44.5
Lecithin	18.6	40.6
Phosphatidyl ethanolaime	17.4	40.0
Sphingomyelin	16.8	42.6
Cardiolipin	61.5	4.8

Activity was measured in the presence of 0.6 mg/ml histone and 29 μg/ml phospholipid (when added).

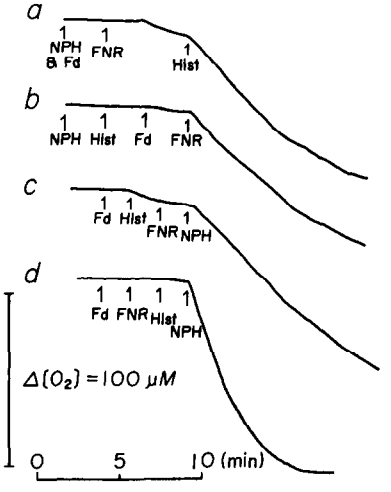

Fig. 1. Effect of the order of addition of the protein components on the O_2-uptake of the FNR–Fd–histone system in the presence of cardiolipin. The reaction mixture originally contained EDTA and cardiolipin, when added. Concentrations: NADPH (NPH) 130 μM; FNR, 0.55 μM; Fd, 2.6 μM; histone (Hist), 0.6 mg/ml; EDTA, 0.29 mM; cardiolipin, *a* none (control), *b* through *d* 29 μg/ml. The experiments were performed only by varying the order of additions.

** Amino acid compositions of these proteins are: Fd(Asp 11, Glu 9, Arg 1, Lys 4, His 1 [9]), Ad(Asp 11, Glu 7, Arg 4, Lys 5, His 3 [10]).

histone caused an increase of turbidity, indicating a formation of macromolecular aggregates made of histone and cardiolipin. To observe the enhanced oxidase activity, an order of addition of these three proteins was found to be important. In fig. 1, curve *a* contains no cardiolipin, and curves *b*, *c*, and *d* contain cardiolipin. When histone was added into the reaction mixture containing cardiolipin prior to either one or both FNR and Fd, no further increase in the oxidase activity was observed (curves *b* and *c*). For a full stimulation, histone must be added after the additions of both FNR and Fd (curve *d*). These results are most likely interpreted as follows. For the enhancement of the oxidase activity, the FNR–Fd complex must be incorporated into the micellar structure of the histone–cardiolipin aggregates. If the aggregates are made before the formation of the FNR–Fd complex, no further enhancement of the activity is observed. The incorporation of the complex into the aggregates may be due to the strong protein–protein interaction between histone and Fd component.

As demonstrated in this paper, the interactions of histone with acidic proteins such as Fd and Ad or with acidic phospholipid are quite evident, and these interactions can occur in physiological circumstances. Of importance is the impression that the degree of ionic interaction between acidic and basic protein components is an important determinant for the catalytic activity of the multiprotein systems. The results obtained here could provide a clue to the elucidation of unknown functions of cooperativity among basic proteins, acidic proteins, and acidic phospholipid in membrane systems.

In this regard, a number of investigators [11–13] reported that basic proteins cause a block in proton transport across mitochondrial membranes and chloroplast membranes in the dark. On the other hand, a direct interaction of histone with electron carriers must also be considered. Recently, Brand et al. [14, 15] have reported that histone inhibits the photosystem 1 activities of isolated chloroplasts from a variety of higher plants but not of cell-free preparations of several algae. They observed that histone causes partial inhibition of indophenol dye photoreduction by chloroplasts but has little effect on indophenol photoreduction by photosystem 2 particles. In any event, basic proteins have effects on membrane-bound enzyme systems.

The exact molecular events of the histone-stimulated NADPH-oxidase activity catalyzed by the FNR–Fd and the AR–Ad systems and the effects of cardiolipin on these systems must remain largely to be elucidated in further studies.

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