DETECTION AND INVESTIGATION OF A PROTEIN FACTOR CONCERNED WITH THE STIMULATION OF DISULFIDE REDUCTASE ACTIVITY BY CYCLIC AMP AND OTHER EFFECTORS

V. I. Kulinskii and L. S. Kolesnichenko

UDC 612.351.1+612.82.015.2

Stimulation of disulfide reductase (DSR) in the supernatant fraction of mouse liver by 3',5'-AMP (10^{-7} M), ATP ($5 \cdot 10^{-5}$ M), Mg^{++} ($5 \cdot 10^{-5}$ M), EDTA ($5 \cdot 10^{-4}$ M), and protamine (5 mg/ml) is mediated by a factor which is readily adsorbed by $BaSO_4$, $Al(OH)_3$, and activated charcoal, and is readily eluted by a tenfold increase in the molarity of the buffer. Barium eluates of the liver and brain mutually and equally restore the effect of 3',5'-AMP when abolished by $BaSO_4$. The adsorbed factor is evidently a protein, for it does not dialyze, it is very thermolabile, and it is easily inactivated by low concentrations of trypsin. The factor restores activation of DSR when abolished or reduced by protein inhibitor of protein kinase (PIPK). The fact that the relationship between the active quantities of PIPK and protein factor (PF) is linear indicates that the PF is similar or identical to protein kinase. The possible biological role of activation of DSR and the hypothesis that a special protamine-sensitive protein kinase exists are discussed.

KEY WORDS: disulfide reductase; mouse liver; cyclic nucleotides; protein kinase.

Disulfide reductase (DSR) in the supernatant of mouse liver is activated by 3',5'-AMP [2, 3], ATP, Mg^{++}, protamine [2], and EDTA. All these effects are abolished by treatment of the DSR enzyme preparation with adsorbents and with protein inhibitor of protein kinase (PIPK).

The object of this investigation was to test the hypothesis that activation of DSR takes place through the intermediary of a special protein factor (PF) and to study some of its properties.

EXPERIMENTAL METHOD

Experiments were carried out on female CBA mice aged 3-5 months. Activity of DSR was determined [3, 4] in the 27,000-g supernatant of the liver, with 5,5'-dithio-bis-2-nitrobenzoate in a concentration of $6 \cdot 10^{-5}$ M and NADPH in a concentration of $1 \cdot 10^{-4}$ M. Protein was determined by Inoue's method [8]. The additives were preincubated with the enzyme preparation for 10 min at 30°C. The PF was adsorbed on 2.9 mg $BaSO_4$, 5 mg $Al(OH)_3$, and 1 mg activated charcoal to 1 ml of supernatant. Elution from the adsorbents was carried out with 0.1 M Tris-HCl, pH 7.5, in the presence of 1 mmole EDTA (the molarity of the buffer was ten times higher than for adsorption). Dialysis was carried out against 5 mM phosphate buffer with 1 mmole EDTA, pH 7.5, for 20 h at 4°C. The PIPK was obtained from rabbit skeletal muscles and was taken through the first three stages of purification described in [10]. Crystalline trypsin (Spofa, Czechoslovakia) was added to the "barium eluate" to a concentration of 0.5 μg/ml (1:50 relative to protein of the eluate) and incubated for 15 min at 30°C, after which soy trypsin inhibitor (Reanal, Hungary) was added to a concentration of 5 mg/ml. Under the conditions chosen addition of a mixture of trypsin and its inhibitor directly to the enzyme preparation did not alter the activating effect of 3',5'-AMP or the background DSR activity ($P > 0.1$).

Department of Biochemistry, Krasnoyarsk Medical Institute. (Presented by Academician of the Academy of Medical Sciences of the USSR V. N. Orekhovich.) Translated from Byulleten' Eksperimental'noi Biologii i Meditsiny, Vol. 81, No. 6, pp. 662-665, June, 1976. Original article submitted October 22, 1974.

TABLE 1. Modification of Action of Stimulators of Disulfide Reductase Activity in Supernatant of Mouse Liver (M ± m)

Experimental conditions		Activation of DSR (in nmoles substrate/min/mg protein)				
treatment of enzyme preparation	eluate from $BaSO_4$	3′, 5′-AMP (10^{-7} M)	ATP ($5\cdot10^{-5}$ M)	Mg^{2+} ($5\cdot10^{-5}$ M)	EDTA ($5\cdot10^{-4}$ M)	protamine (5 mg/ml)
—	—	2,65± 0,03*	3,05± 0,04*	3,31± 0,04*	3,05± 0,04*	7,67± 0,75*
$BaSO_4$	—	0,20± 0,20	—0,30± 0,2	0,93± 0,78	0,12± 0,35	3,91± 0,42*
$BaSO_4$	+	2,60± 0,34*	3,72± 0,85*	3,97± 1,00*	3,79± 0,78*	7,94± 1,00*
$Al(OH)_3$	—	0,08± 0,14	0,33± 0,47	—0,10± 0,4	0,41± 0,32	1,06± 0,36*
$Al(OH)_3$	+	2,25± 0,60*	2,57± 0,53*	2,07± 0,41*	1,37± 0,40*	6,44± 0,60*
Charcoal	—	—0,39± 0,3	0,32± 0,40	1,95± 0,37	0,51± 0,43	3,45± 0,60
Charcoal	+	2,65± 0,60*	2,28± 0,56*	2,50± 0,51*	2,65± 0,45*	11,40± 1,19*
55°, 9 min	—	—0,06± 0,2	—0,25± 0,7	—0,60± 0,6	0,30± 0,37	0,18± 1,02
55°, 9 min	+	2,32± 0,43*	2,54± 0,67*	1,91± 0,50*	2,39± 0,41*	4,22± 0,95*
$BaSO_4$	+	0,38± 0,58	0,63± 0,83	—0,48± 0,74	—0,17± 0,85	4,20± 1,28*
$BaSO_4$	+ dialyzed					
2′, 3′-AMP		2,20± 0,60*	2,35± 0,66*	2,20± 0,66*	1,61± 0,45*	10,88± 3,50*
$5\cdot10^{-5}$M	—	0,25± 0,17	0,60± 0,51	0,85± 0,70	3,47± 0,63*	6,60± 1,10
2′, 3′-AMP	+	2,20± 0,54*	2,64± 0,68*	3,08± 0,46*	3,00± 0,89*	10,04± 2,20*
PIPK (5 μg/ml)	—	0,02± 0,30	0,05± 0,08	1,00± 0,44	1,91± 0,45*	6,03± 0,61*
PIPK	+	3,45± 0,90*	3,97± 0,62*	3,78± 0,93*	4,42± 0,90*	11,28± 0,42*

*P < 0.05 compared with initial DSR level.

Legend. In series of 6-12 experiments. Barium eluate contained 2.6 μg protein.

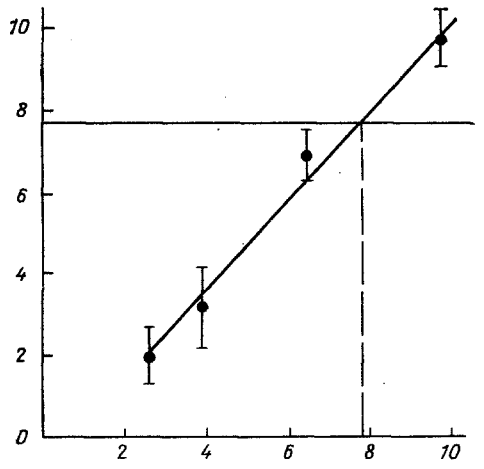

Fig. 1

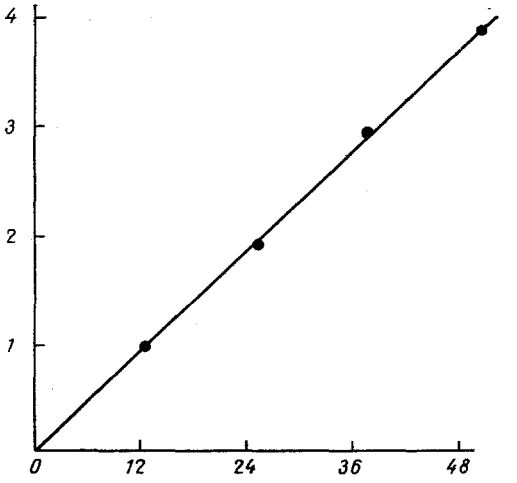

Fig. 2

Fig. 1. Restoration of activating effect of protamine on DSR on addition of barium eluate to enzyme preparation from liver treated with protein inhibitor of protein kinase. Abscissa, quantity of protein in added volume of eluate (in μg); ordinate, activating effect of protamine (5 mg/ml) on DSR (in nmoles) substrate/min/mg protein. Horizontal line indicates effect of protamine without PIPK. A perpendicular is dropped from the point of its intersection with the experimental straight line and it intersects the abscissa at the point corresponding to 8 μg of protein factor. This is equivalent to the quantity of PIPK added to the given sample (62 μg).

Fig. 2. Relationship between active quantities of protein inhibitor of protein kinase and protein factor. Abscissa, amount of PIPK added to sample (in μg); ordinate, amount of PF (in μg) completely restoring activating effect of 3′,5′-AMP on DSR.

EXPERIMENTAL RESULTS

It will be clear from Table 1 that treatment of the DSR enzyme preparation with adsorbents completely abolished the effect of 3′,5′-AMP, ATP, and EDTA and considerably reduced (or abolished) the action of protamine and Mg^{++}. Addition of the "barium eluate" did not change the background DSR activity but it fully restored the effects of all the activators after

treatment with any one of the adsorbents. Additional experiments showed that the "charcoal eluate" possessed the same properties with respect to 3',5'-AMP and noradrenalin. Consequently, all the adsorbents removed the same readily adsorbed and readily eluted factor, concerned in the activation of DSR, from the supernatant.

The results showed that this factor does not possess tissue specificity: Barium eluates of enzyme preparations from the liver and brain mutually and equally restored the effect of 3',5'-AMP after preliminary treatment with $BaSO_4$.

Heating the enzyme preparation, untreated by adsorbents, for 9 min at 55°C completely prevented activation of DSR. The addition of barium eluate to such a heated preparation restored the effect. Consequently, the factor mediating activation of DSR possesses high thermolability. This was confirmed by the fact that the restorative effect of the barium eluate disappeared if it was heated under the same conditions. Prolonged dialysis of the barium eluate did not abolish its ability to restore the effect of the activators. The inability to dialyze and the thermolability of the factor suggested that it is protein in nature. This was confirmed in the experiments by rapid inactivation (15 min, 30°C) of the factor by a low concentration of trypsin.

2',3'-AMP and PIPK completely blocked the activating action of 3',5'-AMP, ATP, and Mg^{++} on DSR. The addition of PF (barium eluate) restored all these effects (Table 1). It is interesting to note that in situations when the inhibitors only reduced (PIPK with protamine and EDTA) or did not change (2',3'-AMP with protamine) the stimulating effect, the addition of PF usually actually increased the action of the activators on DSR (the background DSR activity, however, was unchanged).

This effect could signify that the action of DSR activators was limited by the amount, not of the enzyme itself, but of PF. To test this hypothesis experiments were carried out with the addition of PF to the ordinary enzyme preparation. As a result, the action of 3',5'-AMP increased from 2.36 ± 0.28 to 4.26 ± 1.16 and that of protamine from 7.67 ± 0.75 to 12.40 ± 1.47 nmoles substrate/min/mg protein.

Considering the high specificity of PIPK [11, 12], the restoration of DSR activation through its influence with the aid of PF suggests that PF is similar or identical to protein kinase. This hypothesis is supported by the similar thermolability [5, 11] and tissue nonspecificity [7] of protein kinase. On the gradual addition of PF to enzyme preparations previously mixed with PIPK, the activating effect of both protamine (Fig. 1) and 3',5'-AMP is gradually restored. In this way the quantity of PF equivalent to different concentrations of PIPK can be "titrated." The results of one such titration are given in Fig. 2. A simple linear relationship evidently holds good between the active quantities of PF and PIPK, suggesting stoichiometric interaction between these two proteins. This is an important argument in support of the view that the PF discovered by the writers may be protein kinase. Confirmation of this hypothesis would mean that the protein kinase mechanism can also regulate enzymes of the oxidoreductase class.

The substantial quantitative differences in the change in the effect of protamine compared with the other activators (Table 1) could be explained on the assumption that a special form of protein kinase, sensitive only to protamine, exists in the tissues and that it is less readily adsorbed and is more resistant to PIPK, but equally thermolabile. The hypothesis of the existence of forms of protein kinases with different methods of regulation is in agreement with the presence of two forms of 3',5'-AMP-dependent protein kinase in the rat liver [9] and of a special protamine kinase, insensitive to 3',5'-AMP and not identical with the catalytic subunit of protein kinase, in the brain [8]. Unfortunately the authors of the last work cited did not study the possible activation of this form of protein kinase by protamine.

This possibility that 3',5'-AMP may participate in the regulation of thiol-disulfide metabolism is of great interest, for oxidation of SH groups is one way not only of nonspecific inactivation, but also of quantitative (chemical modification) [7] and qualitative (transformation) [1, 4] alteration of enzyme activity. It can tentatively be suggested that the 3',5'-AMP—protein kinase mechanism can restore SH groups and also, consequently, normal function of the enzymes, and that this may prevent the development of a secondary pathology of metabolism such as is observed, for example, through the transformation of enzymes [1] in cases of vitamin imbalance, malignant disease, and radiation sickness.

LITERATURE CITED

1. V. Z. Gorkin, Vopr. Med. Khim., No. 2, 118 (1972).
2. L. S. Kolesnichenko, in: Disturbances of Metabolism [in Russian], Tomsk (1974), p. 40.
3. V. I. Kulinskii and V. V. Ivanov, Dokl. Akad. Nauk SSSR, 213, 1439 (1973).
4. W. S. Allison, L. V. Benites, and C. L. Johnson, Biochem. Biophys. Res. Commun., 52, 1403 (1973).
5. H. Brostrom, E. M. Reimann, et al., Adv. Enzyme Reg., 8, 191 (1970).
6. I. D. Corbin, E. M. Reimann, et al., J. Biol. Chem., 245, 4849 (1970).
7. H. Holzer and W. Duntze, Ann. Rev. Biochem., 40, 345 (1971).
8. I. Inoue, H. Yamamura, and I. Nishizuka, Biochem. Biophys. Res. Commun., 50, 228 (1973).
9. A. Kumon, K. Nishiyama, et al., J. Biol. Chem., 247, 3726 (1972).
10. O. H. Lowry et al., J. Biol. Chem., 193, 265 (1951).
11. T. R. Soderling, I. P. Hickenbottom, et al., J. Biol. Chem., 245, 6317 (1970).
12. D. A. Walsh, C. D. Ashby, et al., J. Biol. Chem., 246, 1977 (1971).

EFFECT OF BODY VITAMIN K LEVEL ON COLLAGEN METABOLISM IN THE SKIN

P. N. Sharaev, N. G. Bogdanov, and R. N. Yamaldinov

UDC 612.79.015.348:547.962.9] .015.6:577.161.3

In rats with secondary avitaminosis-K induced by Pelentan the collagen content in the skin is reduced and the content of free hydroxyproline increased. The rate of acid hydrolysis of collagen is increased. Vitamin K (Vikasol*) prevents the development of the changes in collagen metabolism.

KEY WORDS: collagen; vitamin K; avitaminosis-K.

Vitamin K and its synthetic analogs have been used with success in the treatment of diseases accompanied by lesions of the connective tissue [4, 7]. The therapeutic value of vitamin K in these diseases and during administration of anticoagulants with indirect action can be explained by its ability to restore disturbances of fibrillogenesis and of the resistance and permeability of the tissues [1, 3, 5, 8, 9]. The state of these tissue functions is known to be largely dependent on collagen and mucopolysaccharide metabolism.

It was accordingly decided to study collagen metabolism in the skin of rats receiving vitamin K (Vikasol*) and its antagonist, Pelentan.

EXPERIMENTAL METHOD

Experiments were carried out on rats weighing 130-150 g, receiving daily intramuscular injections of Vikasol solution in a dose of 10 mg/kg for 10 days or Pelentan by mouth in a dose of 40 mg/kg for 15-20 days, or both preparations together for 20 days. In special experiments rats with avitaminosis-K, at the conclusion of their course of Pelentan, received Vikasol in a dose of 10 mg/kg daily for 8 days. Some rats after developing avitaminosis-K were kept under ordinary conditions. The development of avitaminosis-K was monitored by determining the prothrombin time. The rats were decapitated at the end of the experiments. A weighed sample of skin was homogenized and collagen was extracted from it with hot TCA solution [11]. Hydrochloric acid was added to the resulting extracts (up to a concentration of

*Bisulfite derivative of 2-methyl-1,4-naphthoquinone — Translator.

Department of Biochemistry, Izhevsk Medical Institute. (Presented by Academician of the Academy of Medical Sciences of the USSR V. N. Orekhovich.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 81, No. 6, pp. 665-666, June, 1976. Original article submitted September 15, 1975.