Vol. 191, No. 1, 1993
February 26, 1993

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 95-102

A PROVISIONAL MECHANISM FOR REGULATING THE AMINOACYL-tRNA SYNTHETASES

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Received December 3, 1992

A mechanism is outlined for regulating aminoacyl-tRNA synthetases with a nonheme iron-containing protein serving as the key regulator. This mechanism is formulated from experiments with a complex-bound valyl-tRNA synthetase from yeast that is activated by thiols, by tRNA, and by an iron-containing protein preparation with characteristic spectral properties.

Early in the study of aminoacyl-tRNA synthetases it was noted that their turnover numbers are very low (1), an observation consistent with the careful comparative rate measurements of Berg *et al* (2). Calculations from the latter findings show that, though the rates of aminoacyl-AMP formation are adequate for protein biosynthesis in the cells from which these enzymes are obtained, the corresponding aminoacylation of tRNA is much slower and far from adequate. Numerous inconclusive efforts to find an activator that might increase the low activities to more reasonable levels are listed by Dignam and Deutscher (3). The work described here indicates that in an integrated complex of several synthetases associated with an oxido-reductive regulatory apparatus (4-6) the reduced form of a nonheme iron protein serves as an activator (Scheme I).

The present experiments were performed with a complex-bound valyl-tRNA synthetase from yeast that requires a thiol for activation in a process that is arrested almost instantaneously by arsenite (4). Thus, in the experiment recorded in Fig. 1A, after an activation process was initiated, samples were transferred to arsenite at 15-second intervals and

0006-291X/93 $4.00

the active enzyme accumulated up to sampling time assayed later from its rate of catalysis of ^{3}H-valyl-tRNA formation (4).

Results

Thiol Effects--The experiment of Fig. 1A demonstrates the activation produced by 5 mM DTT with a crude preparation of the enzyme complex. When the process is initiated with an ammonium sulfate precipated paste of the complex and the solution stirred as described in the legend, a synchronous oscillation occurs in the amount of accumulating active synthetase. The oscillation may be due to a cascade-like mechanism (7) that modulates the rate and direction of the activation outlined in

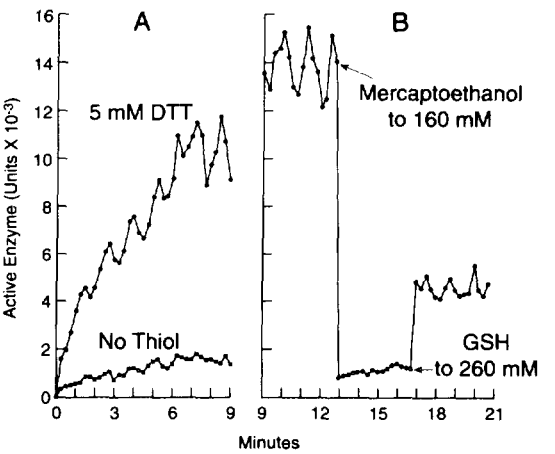
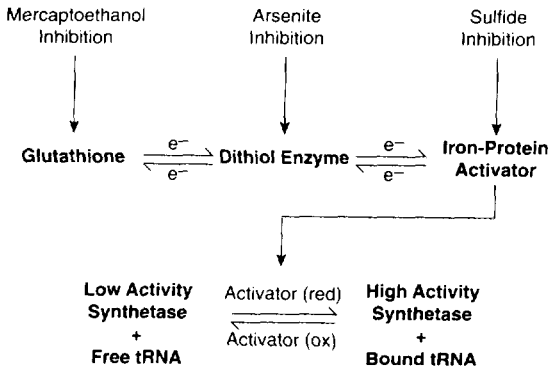


Fig. 1. Reductive activation of complex-bound valyl-tRNA synthetase. The enzyme complex was centrifugally deposited as a 50% saturated ammonium sulfate-insoluble fraction (4) in portions containing 0.9 mg of protein in 1.5 ml Eppendorf tubes and stored at -80°. A. A 0.5 x 8 mm magnetic stirrer was added to an enzyme-containing Eppendorf tube and started to rapidly rotate before adding 400 µl of a reaction mixture containing 50 mM Na bicine (pH 8.3), 1 mM $MgCl_2$, 1 mM ATP, 5 mM DTT, and 30 µg/ml of yeast tRNA (Schwarz/Mann). The temperature was 23°. Samples of 10 µl were transferred at 15 second intervals to 100 µl of 50 mM Tris arsenite-50 mM Tris chloride (pH 8.0), and these solutions, with regulatory change arrested by arsenite (4), then assayed for active enzyme as described (4). The units of activity are the counts per minute of ^{3}H-valyl-tRNA found in the assays. B. This experiment was begun as that of A. except that 600 µl of reaction mixture was used and sampling begun at 9 minutes. Immediately after the sampling at 13 minutes 5 µl of mercaptoethanol (14 M) was added, and just prior to the 17 minute sampling 60 µl of 1.6 M K-GSH-0.16 M mercaptoethanol (pH 8.3) was added. The subsequent assay values were normalized to correct for this dilution.



Scheme I. Outline of a hypothetical mechanism for the reductive activation of aminoacyl-tRNA synthetases. The activation occurs within an integrated complex of several synthetases associated with regulatory proteins.

Scheme I, but it has not been further investigated. The control values in Fig. 1A are presumed to be due to incompletely oxidized endogenous GSH. In the experiment of Fig. 1B the precipitous drop in active enzyme caused by mercaptoethanol and the partial reversal by GSH suggest that tightly bound GSH is essential to maintenance of the active state and that its function is competitively blocked by mercaptoethanol. The activation of crude preparations by DTT and mercaptoethanol in non-inhibitory concentrations would thus occur via reduction of endogenous GSSG.

Following gel filtration of the enzyme complex in the presence of a thiol-binding mercurial the extent of activation by 30 mm DTT is very limited (Fig. 2, lower curves), but GSH and cysteine produce rapid activation to levels dependent on their concentrations up to 0.1 M. Requirements up to 0.3 M have been found with preparations gel filtered in 65% ethylene glycol with no mercurial. One interpretation of these observations is that when tightly bound GSH is removed the structure of the regulatory complex is impaired in a manner that reduces both its affinity and specificity for GSH. It is noteworthy that the activity of a regulatory form of glycyl-tRNA synthetase can also become dependent on GSH or cysteine though unresponsive to DTT or mercaptoethanol (8). Glutathionyl spermidine, first isolated from E. coli (9), has been synthesized by the method of Henderson et al (10) and at 10 mM found to approximate the activity of 10 mM GSH or cysteine with a mercurial filtered complex.

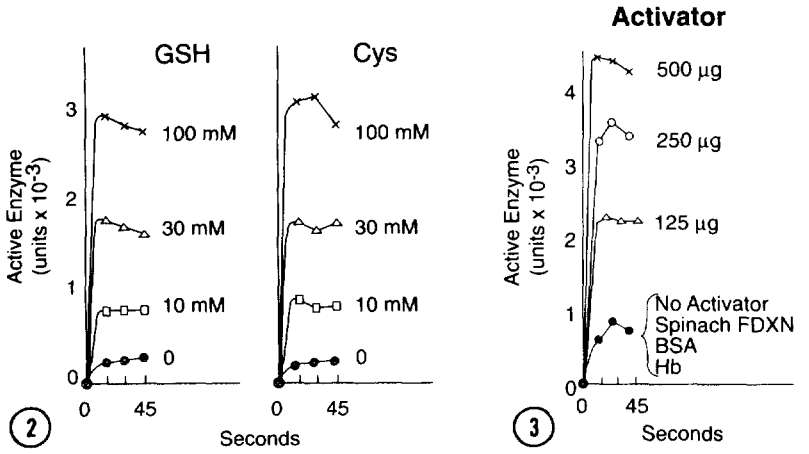


Fig. 2. Requirement for glutathione or cysteine to activate a complex-bound valyl-tRNA synthetase that was gel-filtered in the presence of a mercurial. The enzyme complex was prepared as described (4) except that prior to the freezing step the original extract was mixed with an equivalent volume of 0.03 M hydroxyethyl disulfide in 10% ethanol, a yield increasing addition to the original procedure. The 50% saturated ammonium sulfate insoluble fraction was centrifugally deposited in 1.35 mg portions in 1.5 ml Eppendorf tubes and stored at -80°. The complex in one such tube was dissolved in 0.5 ml of 0.4 M mercaptoethanol-0.1 M Na_2S-0.2 M bicine (pH 8.3) and left at 2° for 16 hours, a step that maximizes the subsequent activability of the enzyme while the reductive activation itself is prevented by the inhibitory actions of mercaptoethanol and sulfide. A 0.35 ml portion of this solution was then centrifugally filtered (19) through 3 ml of Sephacryl S-200 (superfine) that was previously washed with 3 volumes of 0.02 M Na bicine (pH 8.3) containing 0.01 M Na mersalyl (a mercurial also called salyrgan). Sixty-eight microliters of the gel-filtered complex was then mixed rapidly at 23° with 32 µl of a reaction mixture to give final concentrations of 50 mM Na bicine (pH 8.3), 0.5 mM $MgCl_2$, 0.5 mM ATP, 60 µg/ml of yeast tRNA, 30 mM DTT, 400 µg/ml of the reduced activator protein described in the legend to Fig. 4, and the indicated concentrations of glutathione or L-cysteine which had been adjusted to pH 8.3 with KOH. Samples of 20 µl were transferred at 15, 30, and 45 seconds to 100 µl of the arsenite solution described in the legend to Fig. 1, and active enzyme assayed (4).

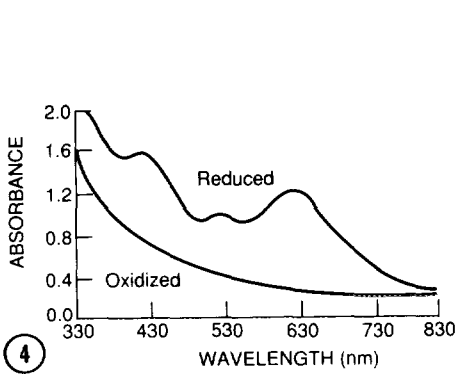
Fig. 3. Activation of aged complex-bound valyl-tRNA synthetase by an iron-containing protein preparation. Ammonium sulfate-precipitated enzyme complex containing 1.35 mg of protein (see legend to Fig. 2) was dissolved in 1.5 ml of a solution containing 0.2 M bicine, 0.1 M Na_2S, 0.4 M mercaptoethanol, and 60 µg/ml of yeast tRNA (pH 8.3), and left at 23° for three days. A 0.35 ml portion was then centrifugally filtered through 3 ml of mercurial-treated Sephacryl S-200 prepared as for the experiment of Fig. 2. Sixty-eight microliters of the gel-filtered aged complex was then mixed rapidly at 23° with 32 µl of a reaction mixture to give final concentrations of 50 mM Na bicine (pH 8.3), 0.5 mM $MgCl_2$, 0.5 mM ATP, 100 mM K-GSH, 30 mM DTT, 60 µg/ml of yeast tRNA, and the indicated quantities per ml of the reduced form of the activator preparation described in the legend to Fig. 4 that had been dialyzed against 0.02 M Na bicine buffer. Tests were also made with 400 µg/ml of spinach ferredoxin (Sigma), BSA, and bovine hemoglobin. Samples of 20 µl were transferred at 15, 30, and 45 seconds to 100 µl of arsenite solution (legend to Fig. 1) and subsequently assayed for active enzyme (4).

The nonheme iron-containing activator preparation--When the complex is aged, the valyl-tRNA synthetase activity diminishes and can be largely restored by a heat stable supplement from yeast. Maximal dependence on this supplement is obtained with solutions of the enzyme complex that have been left highly diluted at room temperature as described in the legend to Fig. 3.

The activity of the supplement is associated with protein that forms a dark green gelatinous precipitate when dialyzed against inorganic sulfide (legend to Fig. 4). The precipitate can be dissolved only in strong solutions of urea. On SDS electrophoresis in a 20% homogeneous polyacrylamide Phastgel the protein material, following Coomassie Blue staining, yields seven bands of similar intensity with molecular weights ranging from 14,000 to 35,000. When the precipitate was dissolved in 10 M urea and reprecipitated four times by dialysis against sulfide (legend to Fig. 4) the composition on gel electrophoresis was unchanged despite a 60% loss of the originally precipitated protein. Two equivalents of iron were found per 22,000 grams of protein by the method of Davis et al (11). Thus all of the polypeptides in the preparation could conceivably contain iron. The visible light absorption spectra of the reduced and oxidized forms of the protein, shown in Fig. 4, are distinguished from corresponding spectra of the nonheme iron-containing ferredoxins (12) by the relatively much stronger absorption of the reduced form.

Neither the reduced or oxidized form of the protein material moves on a column of DEAE cellulose when dissolved in 8 M urea-2 M Tris chloride, and a mixture of the reduced and oxidized forms was completely excluded by Sephadex G-75 in bicine-buffered 8 M urea. In an earlier effort to isolate this activity as an antagonist of sulfide inhibition (5) some of it was chromatographable in the crude state but severe losses and qualitative deterioration occurred as purification proceeded, apparently because of an extreme tendency to aggregate. It is notable, however, that a one-hundred-fold purified preparation showed a 300-400 nm light absorption comparable to that of the oxidized form of Fig. 4 (Fig. 16 of reference 5), indicative of the presence of iron.

Fig. 3 shows the activation response of aged complex to several levels of the activator, added as an opalescent



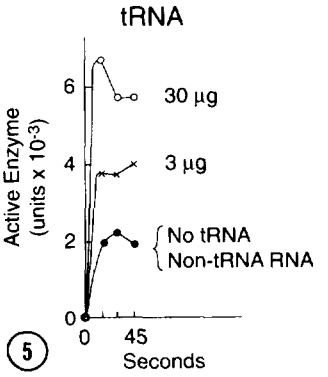


Fig. 4. Visible light absorption by an iron-containing protein activator preparation. Twenty ml of a LiCl-mercaptoethanol heat extract of bakers' yeast (5) was dialyzed two successive times at 23° against 2 liters of a solution containing 0.05 M bicine, 0.025 M Na_2S, 0.001 M spermine tetrahydrochloride, 0.002 M phenylmethylsulfonyl fluoride and 2% ethanol (pH 8.3). A copious dark green precipitate was collected by centrifugation and dissolved in 5 ml of 10 M urea (23 mg of protein representing 10 grams of yeast cake). This solution was dialyzed against 500 ml of bicine-sulfide-spermine (above), the precipitate collected, again dissolved in 10 M urea and dialyzed again. The procedure was repeated until five precipitations had been made, when 9.7 mg of green protein was recovered and dissolved in 4 ml of 8 M urea containing sulfide and bicine as above. This solution was used to measure the spectrum labeled reduced, using the appropriate urea-sulfide-bicine blank. A portion of the solution was dialyzed against a large volume of 0.02 M Na bicine buffer containing 0.1 mM $K_3Fe(CN)_6$, and then against buffer only, whereupon it became opalescent and the green color changed to yellow-brown. To clarify for the spectral measurement labeled oxidized 0.2 gram of urea was added to 0.3 ml. The blank was 0.02 M Na bicine buffer with an equivalent amount of urea added. A Beckman DU-7 spectrophotometer was used. The protein concentration in both cases was 2.4 mg/ml.

Fig. 5. tRNA potentiation of the reductive activation of complex-bound valyl-tRNA synthetase. Fifty percent saturated ammonium sulfate insoluble enzyme complex (4) containing 3 mg of protein in 0.25 ml of 65% ethylene glycol-.02 M imidazole chloride buffer (pH 6.8) was centrifugally filtered through 3 ml of Sephadex G-50 (coarse) containing the same ethylene glycol buffer. Forty microliters of the filtrate were mixed rapidly at 23° with 60 µl of a reaction mixture to give final concentrations of 50 mM imidazole chloride buffer, 1 mM $MgCl_2$, 1 mM ATP, 30 mM DTT, 300 mM K-GSH (pH 6.8), 26% ethylene glycol, and the indicated quantities per ml of yeast tRNA. Non tRNA yeast RNA (Calbiochem) was tested at 30 µg/ml. Ten microliter samples were transferred to 100 µl of arsenite (legend to Fig. 1) at 15, 30, and 45 seconds and assayed for active enzyme (4).

suspension-solution in 0.02 M bicine buffer. No response whatsoever was produced by other proteins tested.

Discussion

Proposed Roles for Glutathione, Dithiol Enzyme, Nonheme Iron Protein and tRNA--The Scheme I mechanism would make the

rate of protein biosynthesis critically dependent on the oxido-reductive state of glutathione, as first proposed by Kosower et al (13). High sensitivity to arsenite is a distinctive feature of certain dithiol-containing flavin enzymes (14). Such an enzyme would be a logical agent for the reduction of iron-containing protein by glutathione, and its position in the Scheme I sequence would allow arsenite inhibition of the activation process without affecting previously activated synthetase, as observed (4). Regulatory synthetase inhibition by inorganic sulfide (4,5,8) reinforces the concept of an iron-containing activator (Scheme I). Thiols and nonheme iron proteins are well-established collaborators in regulating the enzymes of oxygenic photosynthesis (15).

The dissociation constants of synthetase-tRNA complexes are of the order of 10^{-6} M (16), which signifies a relatively low affinity with a potential for large increase by a suitable activator. The data of Fig. 5 show that a very small concentration of unfractionated yeast tRNA, and very likely a much smaller concentration of $tRNA^{Val}$, can increase the reductive activation of complex-bound valyl-tRNA synthetase that has been gel filtered in the presence of 65% ethylene glycol. This effect presumably occurs through a shift to the right of the equilibrium postulated in the lower part of Scheme I. It should be noted that the activator-dependent enzyme complex described in the legend to Fig. 3 could not be successfully prepared without the stabilizing presence of added tRNA. Thus a high tRNA concentration compensates for activator loss in accord with a dependence of enzyme-tRNA affinity on the activator (Scheme I).

The multiplicity of proteins in the iron-containing preparation could represent a family of closely related activators, specific for different synthetase-tRNA units, but possessing the same chromophoric prosthetic group. This idea is supported by the distinctive character and magnitude of the preparation's visible light absorption. Assuming an average molecular weight of 22,000, on the basis of two iron equivalents per mole, the molar extinctions of the three bands in the visible light absorption spectrum of the reduced preparation (Fig. 4) lie between 11,000 and 19,000, somewhat higher than the range for absorption by known nonheme iron proteins (17,18).

ACKNOWLEDGMENTS. I thank Herbert Tabor for unrestricted access to his laboratory facilities; Dorothy Black for technical assistance; C.-H. Niu and V. Labroo for guidance in synthesizing glutathionyl spermidine, and F.H. Bergmann for valuable discussions when this work was first undertaken.

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