Immunochemical and Enzymatic Studies on Glutamate Dehydrogenase and a Related Mutant Protein from *Neurospora crassa*

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ABSTRACT

ROBERTS, D. B. (University of Cambridge, Cambridge, England). Immunochemical and enzymatic studies on glutamate dehydrogenase and a related mutant protein from *Neurospora crassa*. J. Bacteriol. 91:1888-1895. 1966.—When an investigation was made of the inhibition of *Neurospora* glutamate dehydrogenase by bivalent and univalent antibodies, it was shown that the enzyme inhibition is not complete even with excess antibodies. The residual activity was some three times greater with bivalent antibodies, in spite of the observation that the ratio of inhibiting antibodies to catalytic sites was 2:1 for both types of antibody. Substrates did not affect the inhibition of enzyme activity, nor did antibodies affect the K_m for either substrate. An allosteric mechanism for the inhibition of glutamate dehydrogenase by antibodies is proposed. It was also demonstrated that the mutant protein *am-3* can be activated, to show glutamate dehydrogenase activity, by a number of activators. The requirement for the activation was the presence of a carboxymethyl group. The data suggest that the nonactivated protein has two combining sites for L-glutamate: the catalytic and activating sites. The wild-type enzyme has only one of these sites. Because the activating site is distinct from the catalytic site, an allosteric mechanism was postulated for activation. Inhibition of *am-3* activity by antibodies is achieved either by a mechanism similar to the inhibition of wild-type activity or by the antibodies preventing the activation of the mutant protein. The *am-3* protein can be activated by antibodies. Consequently, there appeared to be a relation between the phenomena of enzyme inhibition and *am-3* activation by antibodies; i.e., they alter the configuration of the catalytic site. This alteration was necessary for the activation of *am-3*, but inhibited the activity of the wild-type enzyme.

The majority of investigations of the immunological relationships between a wild-type protein and a mutant form of the protein go no further than answering the question of whether the mutant produces a protein immunologically similar to the wild type. Few studies consider the extent to which these proteins are immunologically similar.

Previous studies on the nicotinamide adenine dinucleotide phosphate (NADP)-linked glutamate dehydrogenase from *Neurospora crassa* and on mutant forms of the protein have shown something of the immunological relationships that exist between the various forms of the

protein (8). The mutant *am-3* produces a protein that can be activated to show glutamate dehydrogenase activity; it is also immunologically similar to the wild-type protein. This mutant was chosen for further study.

To determine the extent of the similarity that exists between these two proteins, it was necessary to have criteria by which the mutant protein could be compared with the wild-type enzyme. The primary basis of comparison employed was the inhibition of catalytic activity by antibodies. The wild-type enzyme was studied with respect to its inhibition by both bivalent and univalent antibodies to elucidate something of the inhibition mechanism.

An investigation of the activation phenomenon had to precede any comparison between the

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inhibition by antibodies of the wild-type and mutant enzyme activities. From these studies it has been possible to propose a mechanism for the activation of the mutant protein and for the inhibition of both the wild-type and mutant enzyme activities by antibodies.

MATERIALS AND METHODS

Neurospora strains. The wild type (am^+) used was initially derived from strain 74A (6), and the mutant $am-3$ was described by Fincham (2).

Growth of cultures. The cultures were grown on Vogel medium (*unpublished data*) (liquid or with agar) at 25 C, and were supplemented with 5 mM alanine when necessary.

Preparation of extracts. Extracts for purification and immunization were prepared from frozen mycelium in an MSE Atomix blender with Dry Ice (3). Smaller quantities for assay were prepared by grinding with a pestle in a mortar with ground glass and buffer. All extracts were made with 0.05 M phosphate buffer (pH 8.0), with 0.001 M ethylenediamine tetraacetic acid (EDTA) added.

Purification. The enzyme glutamate dehydrogenase (GDH) was purified from the crude extract by the method described by Fincham (3).

Enzyme assay. The enzyme activity was measured by following the reduction of NADP by optical density measurements at 340 m μ . The reaction mixture for the wild-type enzyme was: enzyme, 0.2 ml of 0.5 M sodium L-glutamate, 0.2 ml of 4.8×10^{-1} M NADP (0.2%), made up to 3 ml with 0.05 M tris(hydroxymethyl)aminomethane (Tris)-HCl buffer (pH 8.4). The enzyme assay was carried out in a 1-cm silica cell in a Unicam spectrophotometer, with the cell housing maintained at 35 C by circulating water. One enzyme unit is equivalent to the production of 0.48 μ mole of reduced nicotinamide adenine dinucleotide phosphate (NADPH₂) in the first minute after the completion of the reaction mixture.

The protein produced by the mutant $am-3$ shows little catalytic activity in the above assay system. If, however, the protein is incubated with a high concentration of L-glutamate (133 mM) prior to the completion of the reaction mixture with NADP, comparable activity to the wild-type enzyme is observed. No increase in this activated activity is found after 4 min of incubation with the L-glutamate.

Protein estimations. Protein concentrations were estimated by the method of Lowry et al. (5), with reference to a standard curve prepared with casein.

Preparation of antibodies. The preparation of the antibodies used in this work and their nomenclature have been described previously (8). No difference was observed in antienzyme activity between serum and purified γ -globulin prepared from that serum. In the majority of experiments designed to compare the effect of univalent and bivalent antibodies, purified γ -globulin was used as the bivalent antibody preparation. In those experiments where whole serum was used, there was nothing to suggest that a comparison between the behavior of whole serum and univalent antibodies was not valid.

Univalent antibodies were prepared by the method of Porter (7). Fragments I, II, and III were not separated prior to use in experiments. Univalent antibodies were judged to be present because of the ability of the preparation to inhibit catalytic activity but not form precipitin bands on double-diffusion plates. The same preparation was able to block precipitin-band formation between the antigen and bivalent antibody.

RESULTS

Effect of bivalent and univalent antibodies on GDH. Initial studies by Roberts and Pateman (8) indicated that anti-GDH serum possessed both neutralizing and precipitating antibodies.

A comparison between the effect of bivalent and univalent antibodies is shown in Fig. 1. Both types of antibody inhibited the catalytic activity, but the extent of inhibition was different. The residual activity with excess univalent antibodies was three times greater than with excess bivalent antibodies.

The residual activity is present in the enzyme-

FIG. 1. Inhibition of glutamate dehydrogenase activity by bivalent and univalent antibodies. Symbols: O, 0.05 ml of crude wild-type (100 enzyme units) extract incubated with bivalent antibody solution from serum B-1-9 (ninth bleed from rabbit 1 in series B; this serum nomenclature used throughout) in 0.05 M Tris-HCl buffer (pH 8.4) at 37 C; ●, 0.05 ml of the same extract incubated with univalent antibody solution (B-1-8). The antibody-enzyme mixture was incubated for 20 min, and the reaction was started by the addition of a mixture of the substrates, bringing the final volume to 3 ml.

antibody complex. This activity is lost when the enzyme-bivalent antibody precipitate is removed by centrifugation, and on double-diffusion plates the enzyme-bivalent antibody precipitin band stains for catalytic activity after uncomplexed enzyme has been washed away (8). A protein with a molecular weight three times greater than that of GDH (sucrose density method) was observed to have catalytic activity after the enzyme had been treated with univalent antibodies. This high molecular weight protein was thought to be the GDH-univalent antibody complex (Roberts, unpublished data).

Michaelis constants with and without serum. The Michaelis constants for L-glutamate and NADP were determined in the presence and absence of serum (Fig. 2 and 3). A statistical

FIG. 2. K_m values for L-glutamate in the presence and absence of serum. Symbols: ●, Lineweaver-Burk plot for the mean of five experiments with serum; 0.005 ml of purified wild-type enzyme was incubated with 0.01 ml of serum B-1-7 (see Fig. 1) in 2.8 ml of 0.05 M Tris-HCl buffer (pH 8.4) for 20 min at 37 C. The reaction was started by a mixture of NADP and the different concentrations of L-glutamate. ○, Lineweaver-Burk plot for the mean of five experiments without serum; the reaction was started by a mixture of NADP and L-glutamate. Formulas: $1/S = (1/M \times 10^{-6} \text{ L-glutamate})$; $1/V = (1/V \times 10^{-3})$ where V is the activity in enzyme units.

FIG. 3. K_m values for NADP in the presence and absence of serum. Symbols: ●, Lineweaver-Burk plot for the mean of four experiments with serum; 0.005 ml of purified wild-type enzyme was incubated with 0.01 ml of serum B-1-6 (see Fig. 1) in 2.8 ml of 0.05 M Tris-HCl buffer (pH 8.4) for 20 min at 37 C. The reaction was started by a mixture of L-glutamate and the different concentrations of NADP. ○, Lineweaver-Burk plot for the mean of four experiments without serum; the reaction was started by a mixture of NADP and L-glutamate. Formulas: $1/S = (1/M \times 10^{-6} \text{ NADP})$; $1/V = (1/V \times 10^{-3})$ where V is the activity in enzyme units.

analysis of the results was carried out by N. S. Howard (Roberts, Ph.D. Thesis, 1964) and showed no significant difference due to the presence of serum.

The K_m for L-glutamate in the absence of serum was 28.2 mM and the 95% confidence limits were 25.4 and 31.0 mM. In the presence of serum, the K_m was 21.5 mM, with confidence limits of 15.5 and 29.0 mM. The K_m for NADP in the absence of serum was 39.7 μ M, and the confidence limits were 35.7 and 45.9 μ M. In the presence of serum, the K_m was 34.2 μ M with confidence limits of 28.4 and 41.6 μ M.

Ratio of antigenic sites to catalytic sites. The data presented in Fig. 1 were used in the following

formula (1):

$$\log \left(\frac{V}{V_A} - 1 \right) = \log K_A + r \log A$$

where *V* = velocity of the enzyme reaction without antibodies; *V_A* = velocity of the enzyme reaction with *A* of antibodies; *K_A* = dissociation constant for the enzyme-antibody complex; *r* = molecules of antibody complexed with one molecule of enzyme or with one catalytic site.

A regression analysis of the data (Fig. 4) gives *r* as equal to 2.16 for bivalent antibodies and 2.11 for univalent antibodies. These figures show the ratio of inhibiting antibodies to catalytic sites to be 2:1 for both types of antibodies.

Activation of *am-3*. The activation of the altered GDH produced by the mutant *am-3* was reported by Fincham (3). A comparison was made of the effects of a number of organic compounds on the catalytic activity of the wild-type and *am-3* extracts. The results of this survey are given in Table 1.

The effect of the concentration of the activator on the activation was studied (Fig. 5). Although the same maximal activation was obtained with both trisodium citrate and sodium L-glutamate,

FIG. 4. Ratio of antigenic sites to catalytic sites. *V* is the activity of 0.05 ml of wild-type extract. *V_A* is the activity of the wild-type extract after 20 min of incubation with *A* ml of antibody solution. Symbols: ○, with bivalent antibody solution; ●, with univalent antibody solution.

TABLE 1. Activation of *am-3**

Activator†	Activity (enzyme units)	
	Wild type	<i>am-3</i>
Tris-HCl buffer.....	91	4
L-Glutamic acid sodium salt.....	99	40
Trisodium citrate.....	102	79
Sodium acetate.....	94	18
EDTA.....	98	40
Glycine.....	90	0
L-Cysteine hydrochloride.....	96	1
L-Histidine monohydrochloride....	77	25
Saturated solution of 2'2' di- pyridyl.....	6	0
L-Aspartic acid.....	110	43
Glutamine.....	39	2
L-Proline.....	89	0
L-Leucine.....	87	4
D-Glutamic acid.....	41	38

* The pH of the activator was adjusted to 8.4 if necessary. An 0.05-ml amount of extract was incubated with the activator for 4 min prior to the addition of NADP, and 2 min later the L-glutamate was added. In the case of activation by L-glutamate, the NADP was used to start the reaction. In no case was reduction of NADP observed in the absence of L-glutamate.

† All activator compounds were concentrated 0.05 M, except 2'2' dipyridyl.

the concentration of trisodium citrate required for maximal activation was much less than the concentration of L-glutamate.

K_m values for activated *am-3* protein. The *K_m* for L-glutamate for a crude extract of *am-3* was determined after the extract had been incubated for 4 min with either 100, 200, or 300 mM trisodium citrate. After activation, NADP was added and the reaction was started 2 min later by the addition of the L-glutamate. The *K_m* values with the above concentrations of activator were, respectively, 58.3, 39.1, and 33.7 mM. The *K_m* for the wild-type protein under the same conditions was 28.2 mM. The *K_m* for *am-3* after full activation is not significantly different from this.

Determination of the number of L-glutamate combining sites. Wild-type and *am-3* extracts were assayed after 4 min of incubation with different concentrations of L-glutamate, the final concentration in the reaction mixture being the same as that used in the initial incubation period. In each case, the reaction was started by the addition of NADP. The reciprocal of the velocity of the reaction was plotted against the reciprocal of the L-glutamate concentration and against the reciprocal of the square of the L-glutamate concentration

[Fig. 6a (*am*-3) and 6b (wild type)]. Figure 6c (activated *am*-3) shows the results for a similar experiment with *am*-3, in which the protein was fully activated with trisodium citrate and then assayed with various concentrations of L-glutamate. In the first experiment with the nonactivated *am*-3, the plot of the reciprocal of the velocity against the reciprocal of the substrate concentration can only be approximated by a parabola, whereas the plot of the reciprocal of the velocity against the reciprocal of the square of the substrate concentration yields a straight line, suggesting two L-glutamate combining sites per catalytic site. In the second two experiments dealing with active protein, the first plot yields a straight line and the second plot a curve, suggesting only one L-glutamate combining site per catalytic site.

Preincubation of enzyme with substrate. The extent of inhibition of glutamate dehydrogenase by serum is dependent on time (8). The results shown in Fig. 7a and b are those for experiments in which enzyme has been incubated with either bivalent or univalent antibodies for different times in the presence and absence of the substrates. The reaction was started by the addition of either a mixture of the substrates or the

FIG. 5. Activation of *am*-3. A 0.005-ml amount of a crude extract of *am*-3 was activated with different concentrations of activator (L-glutamate or trisodium citrate) by 4 min of incubation in 2 ml of 0.05 M Tris-HCl buffer (pH 8.4). The reaction was started by a mixture of L-glutamate, NADP, and trisodium citrate in 1 ml of the same buffer, bringing the final volume to 3 ml and the final reaction mixture in all experiments to 100 mM L-glutamate and 66 mM trisodium citrate. Symbols: O, trisodium citrate; ●, L-glutamate.

FIG. 6. Determination of the number of L-glutamate combining sites. (a) V is the activity of 0.1 ml of a crude extract of *am*-3 activated by 4 min of incubation with different concentrations of L-glutamate and assayed with the activating concentration of L-glutamate. The reaction was started by the addition of NADP. (b) V is the activity of 0.025 ml of a crude extract of the wild type assayed with different concentrations of L-glutamate. (c) V is the activity of 0.05 ml of a crude extract of *am*-3 activated by 4 min of incubation with 200 mM trisodium citrate and assayed with different concentrations of L-glutamate. S is the L-glutamate concentration (millimolar). Symbols: O, $(1/V \times 10^2)$ against $(1/S \times 10^4)$; ●, $(1/V \times 10^2)$ against $(1/S^2 \times 10^5)$.

missing substrate. In no case was the inhibition of glutamate dehydrogenase activity by antibodies affected by the presence of either of the substrates, even though the final substrate concentration was five times the K_m for that substrate.

Similar experiments were carried out on the *am*-3 protein (Fig. 8). (i) The enzyme was incubated with serum, activated by incubation with L-glutamate, and the reaction was started by the addition of NADP. (ii) The enzyme was incubated with a mixture of both serum and L-glutamate before the addition of the NADP. (iii) The enzyme was incubated with L-glutamate prior to the addition of serum, and again the reaction was started by the addition of NADP. The inhibition pattern with increasing amounts of

serum was markedly different in the first experiment, where the serum was acting on the non-activated enzyme, compared with the second two experiments, where the serum was acting on activated *am*-3 protein. In the second case, the inhibition pattern was similar to that observed for wild-type enzyme, but, in the first case, inhibition was much more effective at higher serum concentrations.

Activation of *am*-3 by serum. In addition to activation by substrates, the *am*-3 protein was activated to a low level of activity in the standard assay system, by use of anti-*am*⁺ antibodies. In the *am*-3 assay system with a high concentration of L-glutamate, the antibodies inhibited the catalytic activity as with wild-type extract. However, in the standard assay system with a low concentration of L-glutamate, the *am*-3 protein showed little activity until the addition of the anti-*am*⁺ antibodies, when significant activity was observed. Neither univalent antibodies nor nonimmune serum showed this activation phenomenon (Fig. 9 and 10).

FIG. 7. Preincubation of enzyme with substrates. Symbols: ○, 0.01 ml (200 enzyme units) of purified GDH incubated with 0.005 ml of antibody solution in 2.8 ml of 0.05 M Tris-HCl buffer (pH 8.4) at 37 C, the reaction being started by the addition of a mixture of substrates; ●, GDH incubated with antibody and NADP as above, the reaction being started by the addition of L-glutamate; ▲, GDH incubated with antibody as above, the reaction being started by the addition of NADP. In all cases the final reaction mixture was the same. (a) Serum J-1-2 (see Fig. 1); (b) univalent antibodies prepared from serum J-1-2.

FIG. 8. Preincubation of enzyme with substrate. Symbols: ●, 0.2 ml (120 enzyme units) of a crude extract of *am*-3 incubated with 133 mM L-glutamate in 2.8 ml of 0.05 M Tris-HCl buffer (pH 8.4) at 37 C, and then with different volumes of serum B-1-7 (see Fig. 1); ○, the same volume of *am*-3 incubated as above with serum B-1-7, and then with 133 mM L-glutamate; ▲, the same volume of *am*-3 incubated as above with serum B-1-7 and with 133 mM L-glutamate. The incubation time with the serum was always 20 min, and the incubation with the L-glutamate before the addition of serum was 4 min.

DISCUSSION

A number of models proposed to account for the inhibition of enzyme activity by antibodies fail to account for the data presented for the glutamate dehydrogenase-anti-glutamate dehydrogenase reaction. In this case, the data are more compatible with the suggestion that the antibodies complex with the enzyme and alter the configuration of the enzyme molecule, thus affecting its catalytic activity.

If the antibodies complexed at the catalytic site, residual activity with excess antibodies might be attributable to a high dissociation constant for the antigen-antibody complex. The dissociation constant, however, is too low to account for the residual activity observed (1.1×10^{-9} moles per liter for the bivalent antibodies and 1.0×10^{-9} moles per liter for the univalent antibodies; Roberts, Federation Proc. 24:694, 1965).

Alternatively, steric hindrance might prevent the bivalent antibody molecules from complexing at all the catalytic sites, some remaining unaffected and showing the observed residual activity. One would then expect a lower residual activity with the smaller univalent antibodies where less steric hindrance would be found.

Instead, the residual activity is greater in the presence of univalent antibodies.

Antibody molecules might hinder either partially or completely the access of the substrates to the catalytic site. Incomplete hindrance would be detected by an increase in the K_m for one or both of the substrates in the presence of serum. No such increase was observed.

The residual activity in the case of complete hindrance would be due to antibodies which complexed with the enzyme but did not affect the catalytic activity. Such antibodies, however, could prevent inhibiting antibodies from complexing and blocking the catalytic site. Owing to their smaller molecular weight, fewer univalent antibodies would complex sufficiently close to the catalytic site to block the catalytic activity, thus accounting for the greater residual activity observed. However, the data show that the number of inhibiting antibody molecules per catalytic site is the same for both types of antibodies, 2:1.

Similar studies on lactic dehydrogenase (4) and luciferase (11) have shown a 1:1 ratio for bivalent antibodies only. Smith et al. (10), while working on carboxypeptidase, found a ratio of 1:1 at 30 C and a ratio of 2:1 at 6.4 C, again for bivalent antibodies.

Before studying the inhibition of the activity of

FIG. 9. Activation of *am-3* by serum. Symbols: ○, 0.025 ml of purified *am-3* incubated with 133 mM L-glutamate and different volumes of serum B-1-7 (see Fig. 1) in 2.8 ml of 0.05 M Tris-HCl buffer (pH 8.4) at 37 C for 20 min; ●, the same amount of *am-3* incubated as above with 33 mM L-glutamate and different volumes of serum B-1-7; ▲, the same volume of *am-3* incubated as above with different volumes of nonimmune serum. In the first two instances, the reaction was started by the addition of NADP; in the last case, by addition of a mixture of the two substrates.

FIG. 10. Activation of *am-3* by serum. Symbols: ○, 0.05 ml of *am-3* extract incubated with different amounts of bivalent antibody solution in 2.8 ml of 0.05 M Tris-HCl buffer (pH 8.4) at 37 C for 20 min (serum B-1-7; see Fig. 1); ●, the same volume of *am-3* incubated as above with univalent antibody solution prepared from serum B-1-8. In both cases the reaction was started by the addition of a mixture of the two substrates. The final concentration of L-glutamate was 33 mM. The two antibody solutions showed comparable inhibition of wild-type activity.

the mutant *am-3* protein by antibodies, the activation of the protein to show catalytic activity was examined.

The spectrum of the activators of the altered protein produced by *am-3* suggested that activation might be due to the chelating properties of the activators. However, the chelating agents dipyriddy and 8-hydroxyphenanthroline did not activate the mutant protein; neither did many amino acids with strong chelating properties. With the exception of histidine, all the activators possess a carboxymethyl group which is proposed as the general activating group. The extent of activation could not be attributed to the number of carboxymethyl groups on the activator molecule.

D-Glutamate activated *am-3* protein but inhibited the wild-type enzyme activity; glutamine also inhibited the wild-type activity. These compounds probably act as competitive inhibitors.

The results given in Fig. 6a show that a plot of the reciprocal of the activity of the *am-3* protein against the reciprocal of the substrate concentration yields a curve which can only be approximated by a parabola. However, when the

reciprocal of the square of the substrate concentration is used, a straight line is obtained, suggesting that there are two binding sites for L-glutamate per catalytic site. Similar results were obtained for *Neurospora* isocitric dehydrogenase by Sanwal, Zink, and Stachow (9). The results of experiments with the wild-type enzyme and with the *am-3* protein fully activated with trisodium citrate suggest only one binding site for L-glutamate per catalytic site.

It seems as though one of the two binding sites for L-glutamate on the *am-3* molecule is the activating site, which is distinct from the catalytic site and is absent in the wild-type enzyme. Presumably, trisodium citrate and all activators complex at this second site on the *am-3* molecule.

The K_m for L-glutamate for the fully activated *am-3* protein is not significantly different from the K_m for L-glutamate for the wild-type enzyme. This evidence is in agreement with the hypothesis that the catalytic site on the *am-3* molecule after activation is the same as the catalytic site of the wild-type enzyme. This change in the catalytic site of the mutant protein is brought about by an activator complexed at a site other than the catalytic site, causing a change in the configuration of the catalytic site.

Substrates do not affect the inhibition of wild-type enzyme activity by antibodies, but the inhibition of *am-3* activity is substrate-dependent. The data shown in Fig. 8 can be considered as demonstrating the effect of antibodies on activated and nonactivated *am-3* protein. In the experiments where the protein has been preincubated with L-glutamate (an activator) or incubated together with L-glutamate and serum, the antibodies are acting on activated *am-3* protein which is inhibited as the wild-type enzyme. In the experiment where the *am-3* protein is incubated with serum prior to activation, the inhibition pattern suggests that the antibodies prevent activation, either by preventing the activator from complexing with the mutant protein or by preventing the postulated change in the configuration of the catalytic site.

The inhibition of glutamate dehydrogenase activity by serum and the activation of *am-3* protein by a number of activators are similar phenomena in that each involves the alteration of the existing catalytic site; one reduces the activity and the other increases it. Serum prepared against the wild-type enzyme activates the *am-3* protein. The serum brings about the change in the configuration of the catalytic site of the mutant protein necessary to restore partial activity. Further addition of serum inhibits this, presumably by further changing the configuration of the catalytic site. In the active *am-3*, where the

catalytic site has already been altered, addition of serum inhibits the activity.

Univalent antibodies do not activate *am-3* protein, but they are also not as efficient at inhibiting the wild-type enzyme (greater residual activity). Both these observations could be due to the reduced efficiency of the univalent antibodies compared with the bivalent antibodies in altering the configuration of the catalytic site.

It seems, therefore, as though inhibition of the enzyme activity of both the activated *am-3* protein and the wild-type protein by antibodies is essentially the same. With the *am-3* protein, there is, however, the additional observation that the antibodies interfere with activation.

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