Change in subunit composition of the iron protein of nitrogenase from *Rhodospirillum rubrum* during activation and inactivation of iron protein

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The subunit composition of the Fe protein of nitrogenase from *Rhodospirillum rubrum* during activation and inactivation was investigated. It was found that the upper subunit (on gel electrophoresis) of the two-subunit Fe protein was converted into the lower subunit during activation *in vitro*. When the Fe protein was inactivated *in vivo* by the addition of NH_4Cl and α-oxoglutarate to the cells, a phosphate-labelled upper band appeared. During activation *in vitro* by the activating enzyme, some of the phosphate of the upper band remained with the protein and appeared in the lower band. Activations *in vitro* were performed on inactive Fe protein obtained from cells grown with glutamate as the nitrogen source. Both native and oxygen-denatured Fe protein exhibited the loss of upper band during treatment with activating enzyme.

The nitrogenase enzyme system of the photosynthetic prokaryote *Rhodospirillum rubrum* is similar to the more studied nitrogenase systems from *Azotobacter vinelandii*, *Clostridium pasteurianum* and *Klebsiella pneumoniae* (see reviews by Emerich & Burris, 1979; Mortenson & Thorneley, 1979). The system consists of an iron-containing protein (the Fe protein) and a larger iron- and molybdenum-containing protein (the MoFe protein). Both proteins have been purified and characterized (Ludden & Burris, 1978; Nordlund *et al.*, 1978). The synthesis of nitrogenase proteins is also repressed by NH_4^+ and O_2, as in other systems (E. Triplett & P. W. Ludden, unpublished work).

R. rubrum and other non-sulphur purple photosynthetic bacteria also exhibit an additional mode of nitrogenase regulation that is not seen in other systems. If NH_4^+ is added to cells which are actively fixing N_2, whole-cell nitrogenase activity is rapidly switched off (Gest & Kamen, 1949; Shick, 1971; Neilson & Nordlund, 1975; Yoch & Cantu, 1980; Sweet & Burris, 1981). Sweet & Burris (1981) have

studied this switch-off carefully and determined that the switch-off occurs when cells have been continuously exposed to some source of NH_4^+, either through N_2 reduction to NH_4^+ or because of low concentrations of NH_4^+ in the medium. Cells that are starved for NH_4^+ do not exhibit the switch-off. The biochemistry, physiology and genetics of this system of regulation in photosynthetic bacteria have been the object of considerable investigation in the past few years (Ludden & Burris, 1976, 1978, 1979; Nordlund *et al.*, 1977; Zumft & Castillo, 1978; Carithers *et al.*, 1979; Wall & Gest, 1979; Yoch & Cantu, 1980; Sweet & Burris, 1981; Hallenbeck *et al.*, 1982). An effect of NH_4^+ on the activity of nitrogenase activity in cells of *Azotobacter vinelandii* has also been observed (Kleiner & Kleinschmidt, 1976; Laane *et al.*, 1980). This effect does not appear to be the same as that described for photosynthetic bacteria.

When *R. rubrum* is grown with glutamate as the N source, cell extracts yield an inactive form of nitrogenase even though the enzyme was active *in vivo* as determined by the acetylene-reduction assay. This inactivity is due to a covalent modification of the Fe protein (Ludden & Burris, 1979). The known components of this modifying group are one phosphate, one pentose molecule and one adenine-like molecule per protein dimer (Ludden & Burris, 1979; Ludden *et al.*, 1982). A part of the covalent modifying group can be removed by an activating enzyme which is isolated from membranes of *R.*

rubrum (Ludden & Burris, 1976; Nordlund *et al.*, 1977). Removal of the modifying group results in activation of the Fe protein (Ludden & Burris, 1979). When *R. rubrum* is grown with limiting ammonia as the N source, active crude extracts are obtained in the absence of activating enzyme (Yoch & Cantu, 1980), and the active, unmodified form of the enzyme can be purified (Ludden *et al.*, 1982). We have previously demonstrated that the inactive modified form of the protein can be separated into two subunit forms on polyacrylamide gels containing SDS (Ludden & Preston, 1980; Ludden *et al.*, 1982). If Fe protein is purified from cells grown in the presence of ^{32}P, only the upper band on SDS/polyacrylamide gels can be shown by auto-radiography to be labelled (Ludden & Preston, 1980; Ludden *et al.*, 1982). Cells grown with limiting ammonia show a single major band corresponding to the lower band of inactive protein and a trace of the upper band. The upper and lower bands are thought to be identical peptides which differ only by the covalent modification of the upper band by the pentose, phosphate, adenine-like molecule group (Ludden *et al.*, 1982). In the present paper the changes in subunit structure of the Fe protein are investigated during activation *in vitro* and inactivation *in vivo*.

Materials and methods

Purification of enzymes

$Rr2_i$ was used for activation studies *in vitro* and purified by a method described previously (Ludden & Burris, 1978). Activating enzyme was purified by the method described by Ludden & Burris (1976). Rr1, used in assays, was purified as described by Ludden & Burris (1978).

Assays in activation in vitro

Activation mixtures contained $900 \mu g$ of $Rr2_i$, $20 mM$-$MgCl_2$, $0.5 mM$-$MnCl_2$, $5 mM$-$Na_2S_2O_4$, $50 mM$-Tris/HCl, pH 7.7, and $100 \mu g$ of purified activating enzyme in a total volume of $210 \mu l$. When activation of O_2-denatured enzyme was being assayed, $Rr2_i$ was denatured by shaking in air for 10 min at $30°C$ before adding to the reaction mix. When activation of native enzyme was assayed, $Rr2_i$ was added after the mixture had been made anaerobic and $Na_2S_2O_4$ had been added; the activation mixture of native enzyme also included $5 mM$-ATP, $20 mM$-phosphocreatine and 0.5 unit of creatine kinase. Activations were always started with the addition of activating enzyme. Samples were removed at various times after the initiation of the assay and quenched into the SDS cocktail described by Laemmli (1970). The subunit composition of each sample was analysed on polyacrylamide gels containing SDS as described by

Laemmli (1970). Each sample applied to the gel contained $2.5 \mu g$ of protein.

The ratio of upper and lower subunits was measured by scanning the Coomassie Blue-stained gels on a densitometer; peaks on the recorder tracing of the densitometer scan were quantified by tracing the peaks on to bond paper, cutting the peaks out and weighing the paper. Results are expressed as the percentage of total peak area (upper-band peak plus lower-band peak) for each peak versus time of activation.

In experiments when activation of native protein was investigated, samples of the activation mixture were also taken for assays of enzymic activity of the Fe protein. Samples containing $100 \mu g$ of Rr2 were taken and injected into an anaerobic acetylene-reduction assay mixture, which included $50 \mu g$ of Rr1, $5 mM$-ATP, $20 mM$-phosphocreatine, 0.5 unit of creatine kinase, $10 mM$-$MgCl_2$, $10 mM$-$Na_2S_2O_4$ and $50 mM$-Tris/HCl, pH 7.7, in a total volume of 0.5 ml. Ethylene produced from acetylene was determined after a 10-min assay. Because no Mn or AE was added to these assays (other than the small amount of carry-over from the activation mix), increases in Fe-protein activity that were seen with increasing time of sampling were taken to indicate that activation of Fe protein had taken place in the incubation mixture.

Preparation of ^{32}P-labelled Fe protein

Fe protein labelled with ^{32}P was prepared from 500 ml batches of cells grown in the presence of 2 mCi of $[^{32}P]P_i$. The ^{32}P-labelled protein was purified as described by Ludden *et al.* (1982). Activations of ^{32}P-labelled proteins *in vitro* were performed just as those of unlabelled proteins were. Analysis of the ^{32}P-labelled proteins was by auto-radiography of SDS/polyacrylamide gels with Kodak XAR film.

Assays of inactivation in vivo

Cells grown on limiting ammonia as the N source under a gas phase of 100% N_2 were used. The cells were grown in the presence of 2 mCi of $[^{32}P]P_i$/ 500 ml batch of cells. Inactivation was initiated by the addition of NH_4Cl and α-oxoglutarate (final concns. 1 mM each). Loss of activity was followed by acetylene-reduction assays at various times after the addition of NH_4Cl and α-oxoglutarate. Switched-off cells (those treated with NH_4Cl and α-oxoglutarate) and control cells that were not switched off were collected and lysed. The extent to which the cells were switched off was measured by the extent to which the activity of extracts was enhanced by the addition of purified activating enzyme. Fe protein from these extracts was purified as described by Ludden *et al.* (1982). The purified proteins

were analysed on SDS/polyacrylamide gels, which were stained for protein and autoradiographed.

Results

Fig. 1 shows the change in subunit structure of $Rr2_i$ during its conversion into $Rr2_a$. The subunit
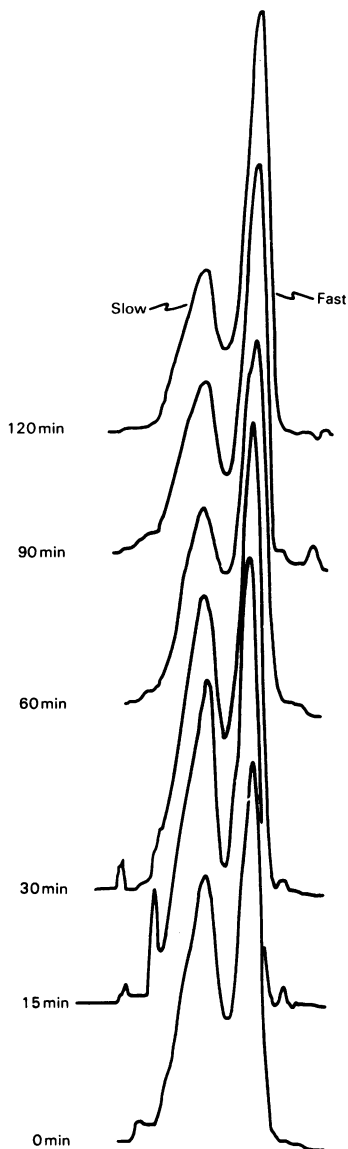
structure of Rr2 was analysed on polyacrylamide gels containing SDS; Fig. 1 shows the densitometer tracings of the gel. Fig. 2 shows the quantification of the data presented in Fig. 1. In this experiment, native (i.e. not O_2-denatured) $Rr2_i$ was used and anaerobic conditions were maintained before and during the activation incubation. ATP, Mg^{2+} and Mn^{2+} were also supplied in the activation mixture. Fig. 3 shows that the activity of Rr2 is increasing during the activation; samples of the incubation mixture taken show increased activity in acetylene-reduction assays that lack activating enzyme. Although a small amount of activating enzyme and Mn are carried over to the acetylene-reduction assay mixture from the activation mixture, it is clear from the initial time point that this contribution is minimal.

It has previously been demonstrated that activation of Rr2 can occur in the absence of ATP if the protein is oxidized by phenazine methosulphate before the addition of activating enzyme (Ludden & Burris, 1979). In addition, the AE was shown to be capable of removing the adenine-like molecule of the modifying group from O_2-treated Rr2 (Ludden & Burris, 1979). Therefore it was expected that subunit conversion would also be seen with O_2-denatured $Rr2_i$ in the absence of ATP. This is demonstrated in Fig. 4. This experiment also clearly shows that not

Fig. 1. *Change in subunit structure during activation of Rr2*

Densitometer tracings of a Coomassie Blue-stained gel are shown. Each time point represents the length of time of activation before the sample was quenched in SDS-containing gel 'cocktail'. Gels were scanned at 545 nm; the top of the gel is to the left.
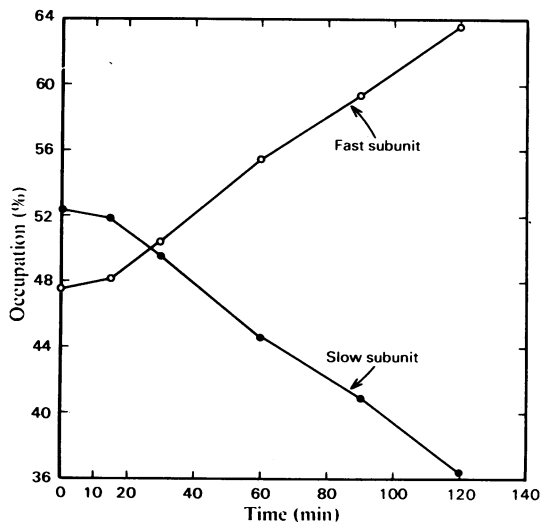

Fig. 2. *Quantification of subunit conversion during activation of Rr2*

The densitometer tracings from Fig. 1 were quantified as described in the Materials and methods section. Percentage occupation is the percentage of the total protein occupying the slow-subunit position (●) or the fast-subunit position (○) on SDS/polyacrylamide gels.
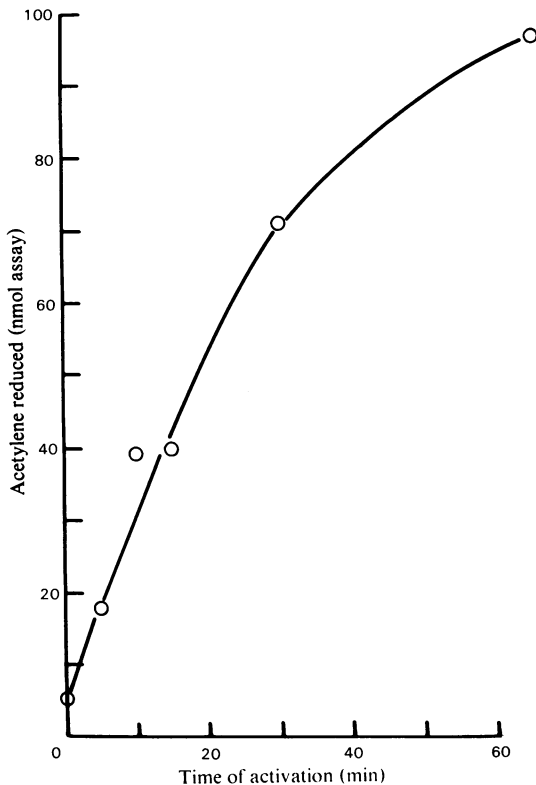
Fig. 3. *Activation of Rr2*

Samples of the activation mixture used in Figs. 1 and 2 were analysed for the ability of Rr2 to participate in acetylene reduction in the absence of AE. Details of the acetylene-assay mixture are given in the Materials and methods section. Each datum point is the average of duplicate 15 min assays.

Fig. 4. *Subunit conversion of O_2-treated $Rr2_i$ by activating enzyme*

$Rr2_i$ was treated with air at 30°C for 10 min. The O_2-treated protein was then added to an anaerobic activation mixture. Samples were taken at the time points indicated and analysed on polyacrylamide gels containing SDS. Quantification of the subunits is as described in the Materials and methods section.

Fig. 5. *Fate of phosphate from ^{32}P-labelled $Rr2_i$ during activation*

^{32}P-labelled protein was treated with AE, Mg^{2+} and Mn^{2+} as described in the Materials and methods section. Tracks 1–8 are described in the text.

only does the upper (modified) subunit decrease, but the lower subunit increases in concentration. At the initial time point, the total area for the upper band plus lower band equals 856 peak units (arbitrary units). At the end of the activation (18 h) there is a total of 795 peak units for the upper band plus lower band, but the upper band has decreased by 275 units and the lower band has increased by 214 units. These measurements were made on samples of the same activation mixture run side by side on a polyacrylamide gel.

Subunit conversion from slow to fast band of O_2-denatured $Rr2_i$ does not proceed as rapidly as activation (subunit conversion) of native $Rr2_i$, indicating that the native enzyme is a better substrate for the activating enzyme.

The fate of the phosphate of the modifying group was also followed during activation. Previous experiments had shown that phosphate was not removed
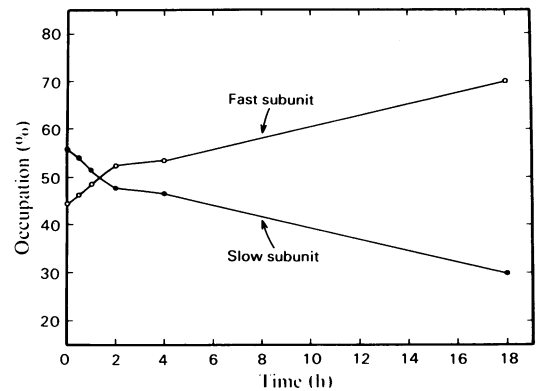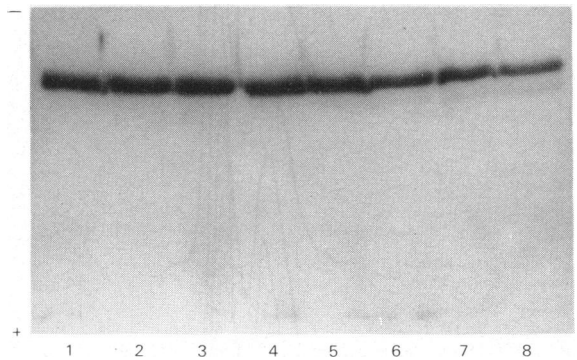
during activation, but that it became susceptible to alkaline phosphatase. Fig. 5 shows the autoradiogram of a gel that was used to measure the fate of phosphate during activation. Tracks 1–4 are controls lacking activating enzyme in the activation mixture: over a 2-h time period no loss of ^{32}P from the upper band occurred and the appearance of ^{32}P in the lower band was not detected. Tracks 5–8 show the effect of activating enzyme on the ^{32}P content of upper and lower bands. The autoradiogram and the densitometer tracings of the
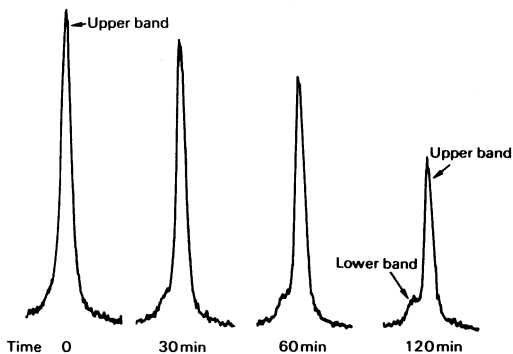
Fig. 6. *Quantification of ^{32}P on upper and lower subunits during activation*

The Figure shows the densitometer tracings of the autoradiogram shown in Fig. 5. 'Time = 0' is a trace of track 4, 'Time = 30 min' is a trace of track 5, 'Time = 60 min' is a trace of track 7 and 'Time = 120 min' is a trace of track 8.

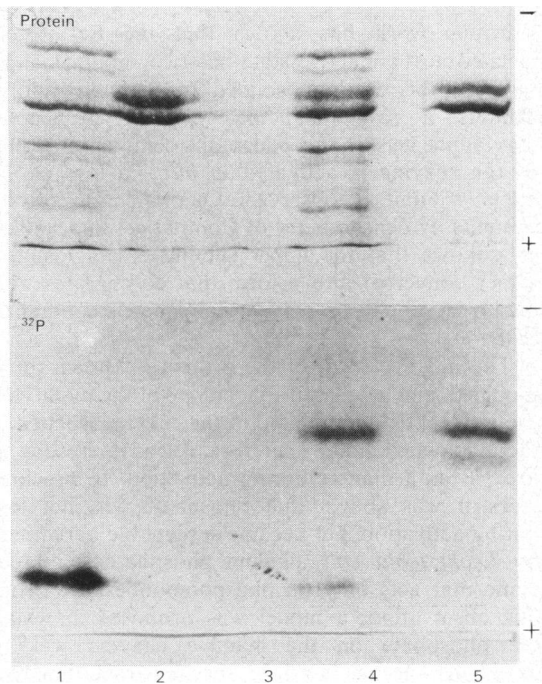

Fig. 7. *Appearance of a ^{32}P-labelled upper band during inactivation of Rr2 in vivo*

The upper portion of the Figure is the Coomassie Blue-stained polyacrylamide gel of active and inactive Rr2. The lower portion of the Figure is the autoradiogram of the gel. Tracks 1–5 are described in the text.

autoradiogram (Fig. 6) show that ^{32}P in the upper band decreases and that some ^{32}P appears in the lower band. It is not possible to conclude that all of the label from the upper band appears in the lower band. It should be noted that the activating enzyme used in this experiment was free of alkaline phosphatase activity and that the addition of phosphatase to the activation mixture resulted in complete loss of the label from the lower band. Treatment of $Rr2_i$ with alkaline phosphatase alone did not result in loss of label from the upper band over the time of the experiment (results not shown).

It is not possible to inactivate $Rr2_a$ *in vitro*; neither the modifying-group donor molecule nor the required enzyme has been discovered. It is possible to inactivate Rr2 *in vivo*, however, and we have tested the incorporation of ^{32}P into Rr2 in this manner. Two 500-ml batches of *R. rubrum* were grown on the ammonia-limited medium of Carithers *et al.* (1979), which yields active extracts. When whole-cell activities of the cultures were 1000 nmol of acetylene reduced/h per ml, one batch was switched off by the addition of 1 mM-NH_4Cl plus 1 mM-α-oxoglutarate. We have found that α-oxoglutarate does not switch off whole-cell activity when added to cultures alone. However, it greatly stimulates switch-off by NH_4Cl. The cells were switched off 41% in 45 min by this treatment. Both the switched-off and the non-switched-off cells were collected, extracts were made and the enzyme was partially purified by chromatography on DEAE-cellulose. The ability of activating enzyme to stimulate activity of purified Rr2 was tested. Rr2 from NH_4^+-treated cells (switched off) was stimulated 33% by activating factor, whereas the

non-switched-off cell extract is stimulated less than 10%. This indicates that more of the NH_4^+-treated Rr2 is switched off. The two batches of Rr2 were then purified and analysed for subunit composition and incorporation of ^{32}P into subunits of Rr2 on polyacrylamide gels containing SDS (Fig. 7). Track 1 shows the protein stain and autoradiogram of Rr2 from cells that are not switched off. Track 2 is a standard containing purified $Rr2_i$ from glutamate cells. Track 4 is the switched-off Rr2 and track 5 is a standard of ^{32}P-labelled $Rr2_i$. It is clear from these results that the active enzyme (track 1) contains only the lower band and that no label is seen in the Fe protein (some contaminating label is seen at the front). When the enzyme is inactivated *in vivo*, the upper protein band appears and it is labelled with ^{32}P (track 4).

Discussion

The data in this paper show that the subunit structure of the Fe protein can be used in determining if the protein is in the active or inactive form.

Previous work has shown that the Fe protein isolated in the inactive state has two subunit bands, whereas the enzyme isolated in the active form exhibits a single subunit type on SDS/poly-acrylamide gels. In this paper, it is demonstrated that as the enzyme is activated *in vitro*, a loss of the upper subunit is observed. Quantification of the subunits by densitometry of Coomassie Blue-stained gels shows that the upper subunit is not lost but rather converted into a form that co-migrates with the lower subunit on polyacrylamide gels containing SDS.

The inactive form of the protein is known to be modified, and at least the phosphate of the modifying group is on the upper band of the enzyme isolated in the inactivated state. Therefore, it was interesting to use ^{32}P as a marker during activation. In previous work it was shown that phosphate was not lost during activation, but became susceptible to removal by *Escherichia coli* alkaline phosphatase, an enzyme that acts only on phosphomonoesters. From this observation, a model was proposed in which the phosphate on the inactive enzyme was in a phosphodiester bond in the following linkage: protein–pentose–phosphate–adenine-like molecule. During activation, the adenine-like molecule is removed and the result is an activated protein with the linkage protein–pentose–phosphate. The exposed phosphate should be accessible to a phospho-monoesterase such as *E. coli* alkaline phosphatase. If this model is true, and activation can be measured by subunit conversion, then it can be expected that phosphate would appear in the lower band during a time course of activation. It is clear that label does occur in the lower band and that the label is phosphatase-labile. It is not clear whether or not a quantitative conversion of phosphate in the upper band into phosphate in the lower band takes place. The densitometer tracings of Fig. 6 would argue against such a quantitative conversion, although determination based on densitometry of film is not as accurate as the direct chemical assays used in the original work.

This work also demonstrates that the addition of NH_4^+ to cells results in the modification of the Fe protein. Because addition of NH_4^+ to the cells also results in loss of whole-cell activity, we conclude that the Fe-protein modification is the reason for loss of nitrogenase activity in whole cells. We have not demonstrated that the entire modifying group is added at once, but it seems likely that this is the case. A change in subunit structure is seen, and studies *in vitro* have shown that this results from the loss of the adenine-like molecule. Phosphate is also added to the upper band, as indicated by the appearance of label in the upper band. Thus it appears that the entire modifying group is added at once. Confirmation of this will require isolation of the donor molecule(s) and purification of the enzyme(s) required for modification.

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