The narGHJI operon encodes the three subunits, α, β, and γ, of the respiratory nitrate reductase complex in *Escherichia coli*. A fourth open reading frame of the operon encodes a putative protein, NarJ, which is not present in purified nitrate reductase, but is required for biogenesis of the membrane-bound complex. NarJ was identified with a T7 expression system and was produced at significantly less than stoichiometric levels relative to the three enzyme subunits. A functional His-tagged NarJ fusion protein was overexpressed from a multicopy plasmid, purified by Ni²⁺ affinity chromatography, and characterized. Western blot analysis with antibodies raised against the fusion protein demonstrated that NarJ remained in the cytosol after assembly of the active membrane complex. The cytosolic αβ complex accumulated in a narJ insertion mutant was rapidly degraded after induction, but was stabilized by NarJ expressed from a multicopy plasmid. Overproduction of the His-tagged NarJ fusion protein in the same mutant led to the formation of an αβ/NarJ complex, which was resolved by Ni²⁺ affinity chromatography. The NarJ protein therefore has the properties of a system-specific (private) chaperone that reacts directly with and modifies the properties of the cytosolic αβ subunit complex, but remains in the cytoplasm after the assembly of the active αβγ complex in the membrane.

Respiratory nitrate reductase in *Escherichia coli* is a membrane-bound electron transfer complex that is composed of three subunits, α, β, and γ encoded by the narG, narH, and narI genes of the narGHJI operon, respectively (1–3). The α and β subunits are hydrophilic proteins that contain molybdenopterin and non-heme iron cofactors and the active site for nitrate reduction. In the membrane complex, these subunits are tightly associated with the hydrophobic γ subunit, a hemeprotein (cytochrome b₇₅₃) that is required for the transfer of electrons from physiological donors through the membrane quinone pool to nitrate (4–6). An αβ dimer has been released from the membrane by heat treatment and purified as an active complex that reduces nitrate to nitrite with artificial electron donors such as reduced methyl viologen (6–8). The αβγ complex has been purified from membranes in the presence of detergents (2, 4, 5); it utilizes quinol analogues as well as reduced viologen dyes as electron donors for nitrate reduction (6).

*The fourth gene product of the narGHJI operon, NarJ, is required for biogenesis of the membrane-bound complex (9, 10), but is not present in the purified preparations of nitrate reductase (2). In a narJ mutant, the γ subunit was inserted normally into the membrane, whereas the αβ complex accumulated in the cytosol in a form that contained a normal complement of cofactors, but was completely inactive (10). In contrast, the αβ complex that accumulated in narJ mutants in the presence of NarJ appeared to express low levels of reduced methyl viologen-nitrate reductase activity (9, 10). These results suggested that, during the biogenesis of nitrate reductase, NarJ may modify the αβ complex or facilitate its interaction with the membrane-bound γ subunit. It was not clear, however, whether NarJ was a subunit of the complex that was lost during the purification procedures or a chaperone-like component that was required only for assembly of the complex. The experiments presented here were undertaken to identify and localize NarJ in the cell and to clarify its possible role in the expression of nitrate reductase.

**MATERIALS AND METHODS**

**Strains and Plasmids—** The *E. coli* strains used in this study, MV1190 (Δ[lac-proAB], thi, supE, ΔsrI-recA)306::Tn10(tetR) [F′ trdA36, proAB, lacI, Δmis] purchased from Bio-Rad, RK4533 (F araI39, ΔargF-lacU169 rpsL150, relA1, F685301, ptsF25, deoC1, rbsR, gyrA4301), K38 (12), and K38 (13), were maintained on L-broth (13). For strains bearing plasmids, the medium was supplemented with 100 μg/ml ampicillin, 25 μg/ml chloramphenicol, or 25 μg/ml tetracycline as required.

Plasmids pSL962 (narGHJI), pMV4 (nar-J), and pES203.1 (narJ) were previously described (2). Plasmid pGPI-2 (Kan⁶, T7 RNA polymerase) and the Amp⁵ T7 expression vectors pT7-7 and pT7-6 were as described by Tubor and Richardson (12). The Cam⁵ T7 expression vector pSU24 was kindly supplied by Valley Stewart (Cornell University). Specific fragments of the narGHJI operon were subcloned into a polycloning site behind the T7 promoter of the T7 expression vectors as diagrammed in Fig. 1A. To construct plasmid pXL241, a 7186-base pair fragment containing the narGHJI genes, isolated on an agarose gel after digestion of plasmid pSL962 with EcoRI (partial) and DraI (complete), was ligated with plasmid pSU24 previously digested with *SmaI* and EcoRI. To construct plasmid pXL243, a 1166-base pair fragment containing the narJ open reading frame, isolated on agarose gel after digestion of plasmid pSL962 with EcoRI and DraI (partial), was ligated with plasmid pSU24 previously digested with *SmaI* and EcoRI. For construction of plasmids pXL742 and pXL762, a 2162-base pair fragment containing the narJL open reading frame, isolated after digestion of pMV4 with PstI and EcoRI, was ligated with pT7-4 and pT7-6, respectively, previously digested with PstI and EcoRI. For construction of plasmids pXL744 and pXL746, a 1559-base pair fragment containing the narJ open reading frame, isolated after digestion of pMV4 with BamHI and EcoRI, was ligated with pT7-4 and pT7-6, respectively, previously digested with BamHI and EcoRI. The structure of each constructed plasmid, isolated from colonies of strain MV1190 transformed to ampicillin resistance, was confirmed by restriction enzyme mapping.

To construct plasmids expressing the His-tagged NarJ fusion protein, a HindIII-EcoRI fragment carrying the narJ open reading frames was subcloned into phagemid pTZ19R (Bio-Rad), and the sequence around the translation initiation codon on narJ was modified by site-
directed mutagenesis to create a NcoI restriction site. This modification (A to G at nucleotide 4 of the open reading frame) resulted in the substitution of Val for Ile at residue 2 of the putative NarJ protein and had no effect on the ability of the plasmid to complement a narJ mutant. The resulting plasmid was digested with NcoI, the ends were blunted with Klenow, and the blunt ends were ligated with pBR322 DNA restriction fragments containing 6 sequential His residues and a enterokinase cleavage site. The resulting plasmid was loaded by gravity, and the unabsorbed proteins were removed by centrifugation at 10,000 × g for 10 min. The supernatant was then adjusted for the 3:1 (NarI/NarJ) ratio of methionine content. The level of NarI (α subunit) even when expressed with the entire operon in amounts that were formed in less than stoichiometric amounts relative to the NarJ operon proteins were also cloned into the T7 expression vector pSU24 (Fig. 1A). NarJ appeared to migrate closely to one of the processed products of the NarJ operon in amounts that were formed in less than stoichiometric amounts relative to the NarJ operon. Western blot analyses employed detection by enhanced chemiluminescence following the protocol described by the manufacturer (Amer sham International). After SDS-PAGE, the proteins were transferred from gels to nitrocellulose paper with a semi-dry Electroblotter (Milli pore). The washing and labeling procedures were as described using horseradish peroxidase-labeled secondary antibodies. Purified nitrate reductase used as standard was prepared previously (2).

RESULTS

Identification of NarJ—The four open reading frames of the narGHJI operon (2, 3) encode proteins with calculated masses of 140 kDa (narG, α subunit), 58 kDa (narH, β subunit), 26.5 kDa (narJ), and 25.5 kDa (narI, γ subunit). To identify NarJ, fragments of the narGHJI operon were cloned into T7 expression vectors (Fig. 1A) and selectively expressed in the presence of [35S]methionine as described under “Materials and Methods.” Parallel constructions were made for each fragment in vectors with the β-lactamase gene oriented in opposite directions so that the processed fragments of β-lactamase could be identified and used as internal controls.

When the narJ and narI genes were expressed together in cells carrying either plasmid pXLL742 or pXL762, two bands corresponding to apparent 29- and 23-kDa proteins were observed (Fig. 1B, lanes 3 and 4) along with β-lactamase and its processed fragments (lane 1). The 23-kDa band was identical to that observed for the narI gene product expressed alone from plasmids pXL744 and pXL764 (lanes 5 and 6), whereas the 29-kDa band, which apparently corresponded to NarJ, migrated closely to one of the processed products of the β-lactamase gene (lanes 1 and 3). Although the putative NarJ protein migrated with an apparent mass that was higher than expected, its expression from pXL762 (lane 4), which contained the β-lactamase gene oriented in the opposite direction, confirmed its identification as the narJ gene product. The level of NarJ appeared to be significantly less than that of NarI when both were expressed from the same plasmid, even when adjusted for the 3:1 (NarJ/NarI) ratio of methionine content. A fragment expressing NarJ only and one expressing all four operon proteins were also cloned into the T7 expression vector pSU24 (Fig. 1A). The selective marker for this vector, cat, encodes a product that is not processed and migrates faster than NarJ on SDS-PAGE, facilitating the identification of NarJ in the selective expression system (Fig. 1C). NarJ appeared to be formed in less than stoichiometric amounts relative to the levels of NarI (γ subunit) even when expressed with the entire operon (fifth through eighth lanes) and even lower when expressed alone (first through fourth lanes). The pulse-chase experiment shown in Fig. 1C demonstrated that the pulse-to-chase ratio of NarJ was not significantly different from those of the other subunits, eliminating a rapid turnover rate as the explanation for the relatively low levels of NarJ.

We conclude that NarJ migrates on SDS-PAGE less rapidly than would be predicted from its mass calculated from the narJ DNA sequence. It appeared to be produced with the T7 expression system at less than stoichiometric levels compared with the other products of the narGHJI operon in amounts that assisted laser desorption ionization mass spectrometry system in the Analytical Chemistry Center.

Protein was determined by the procedure of Lowry et al. (14) or was followed by absorption at 290 nm. SDS-PAGE was carried out on gels prepared and run according to the procedures of Laemmli (15). Whole cells or pellets reacting dissolved in SDS sample buffer and heated for 3 min at 95 °C before loading. If necessary, the undissolved residue from cells was removed by brief centrifugation.

Antisera against the purified NarJ-His6 fusion protein were produced commercially by Bethyl Laboratories, and specific antibodies used for Western blot analyses were purified by the procedure of Oimstein et al. (16) using purified antibodies against the α and β subunits of nitrate reductase were from an earlier study (9) and were purified by the same procedure.

Western blot analyses employed detection by enhanced chemiluminescence following the protocol described by the manufacturer (Amer sham International). After SDS-PAGE, the proteins were transferred from gels to nitrocellulose paper with a semi-dry Electroblotter (Milli pore). The washing and labeling procedures were as described using horseradish peroxidase-labeled secondary antibodies. Purified nitrate reductase used as standard was prepared previously (2).
were not compatible with the development of a facile purification procedure.

Expression and Purification of NarJ as a His-tagged Protein—To facilitate the purification of NarJ, the narJ gene was cloned into a vector to create a fused protein with a polyhistidine tag as described under “Materials and Methods.” The resulting plasmid, pXLIJ-His_{6}, encoded a protein with a 36-residue peptide, containing six tandem His residues and an enterokinase cleavage site, fused to the N-terminal Met of the NarJ sequence. Induction of expression of the fused gene by IPTG in a wild-type strain carrying plasmid pXLJ-His_{6} led to the overexpression of an apparent 33-kDa protein, which was located almost exclusively in the supernatant fraction after high-speed centrifugation of crude extracts (data not shown).

The His-tagged NarJ fusion protein functionally replaced NarJ for expression of nitrate reductase in a narJ mutant. Mutant MD100 produced only α and β subunits and no nitrate reductase activity as the result of an insertion in the narJ gene (9). Transformation of MD100 with pXLIJ-His_{6} or an analogous plasmid, pXLJ-His_{6}, which encoded NarJ as well as the His-tagged NarJ fusion protein, led to the expression of nitrate reductase activities on plate assays (17) similar to those formed with MD100 transformed with plasmids expressing NarJ and NarJ plus NarI (9).

The His-tagged fusion protein was readily purified on a Ni^{2+}-affinity column (Fig. 2A). The overproduced fusion protein appeared to be partially cleaved by endogenous proteases following breakage of cells in the French press (lanes 2 and 3). Much of the overproduced fusion protein and its major cleavage products were absorbed to the Ni^{2+}-resin and eluted by increasing concentrations of imidazole. After most of the contaminating proteins were washed off the column at lower imidazole concentrations (lanes 5–8), the fusion protein and its cleavage products were eluted at 300 mM imidazole (lanes 9–13). The pool of the peak fractions, concentrated and desalted, contained three major components (I–III) on SDS-PAGE (Fig. 2B, lane 2). N-terminal sequence analysis yielded only one sequence, GGSGLHGLAH, which corresponded to that expected for residues 2–11 of the peptide containing the polyhistidine and enterokinase cleavage site motifs. The mass of the largest component (30,639 Da), determined by matrix-assisted laser desorption ionization mass spectrometry, corresponded reasonably closely to that calculated for the fusion protein with the N-terminal Met removed (30,315 Da). Because only one N-terminal sequence was observed for the fraction that was absorbed and eluted from the Ni^{2+}-resin, it seemed most likely that the other two smaller fragments were generated by removal of C-terminal sequences, leaving the N-terminal sequence intact.

Treatment of the pooled purified fraction with enterokinase generated three smaller fragments (Fig. 2B, lane 3). N-terminal sequence analysis revealed only one N-terminal sequence, DRWGSVPLV, which corresponded to the last 5 residues of the fusion peptide and the first 4 residues of the narJ open reading frame. This result indicated that all three peptides contained the same N-terminal sequence after enterokinase cleavage. The mass of the largest fragment resulting from enterokinase cleavage (23,525 Da), as determined by mass spectrometry, corresponded closely to the mass calculated for the cleavage of protein III at the enterokinase site (23,232 Da). These results verify that the purified protein is the expected His-tagged NarJ fusion protein. In extracts, the fusion protein appears to be very sensitive to endogenous proteases, which degrade the protein mainly in the C-terminal region.

The undegraded form of His-tagged NarJ was resolved from most of the partially degraded forms by gel filtration under nondenaturing conditions (Fig. 3A). The affinity-purified protein was resolved into two peaks. Peak I eluted at a position that corresponded to a dimer (66 kDa) and was composed almost exclusively of the undegraded 33-kDa form of the protein (Fig. 3B). Peak II eluted at the monomeric position and appeared to contain both undegraded and degraded forms (Fig. 3B).

Western blots employing antiserum prepared against His-tagged NarJ demonstrated that little, if any, proteolysis of the fusion protein occurred in vivo (Fig. 4). The purified antiserum did not significantly cross-react with other proteins in the cell extracts (lanes 2 and 3), and only the intact protein was visualized in cells that overproduced the His-tagged protein (lanes 4 and 5).

Localization of NarJ—NarJ, produced at relatively low levels under most conditions, could also be visualized on Western blots using the antiserum prepared against the His-tagged protein. In Fig. 5A, low levels of NarJ were observed in extracts of wild-type cells (lane 2) and significantly increased levels in
strains expressing NarJ from multicopy plasmids (lanes 4 and 5). The band identified as NarJ was not present in cells of mutant MD100 (lane 3), which contains an insertionally inactivated narJ gene. When crude extracts from the wild-type strain RK4353 were fractionated by high speed centrifugation, essentially all of the NarJ protein was present in the supernatant fraction (Fig. 5B). Under these same conditions, the nitrate reductase subunits, as assessed by Western blotting with antibodies against the α and β subunits, were located chiefly in the membrane fraction (9).

Formation of an αβNarJ Complex—Although NarJ is not associated with the assembled membrane-bound nitrate reductase complex, it seemed possible that it interacted directly with an intermediate in the assembly pathway. Previous studies with mutants (9, 10) had established that, in the absence of the integral membrane γ subunit, the α and β subunits accumulated as a dimeric complex. In the absence of NarJ, this complex had a normal complement of cofactors (10), but was completely devoid of reduced methyl viologen-nitrate reductase activity. In the presence of NarJ, the complex expressed significant reduced methyl viologen-linked activity, but at very re-

FIG. 2. Isolation of the His-tagged NarJ fusion protein. The fusion protein produced by strain MV1190(pXLJ-His6) was extracted and purified as described under “Materials and Methods.” Equivalent volumes of fractions were subjected to SDS-PAGE (15% acrylamide), and the gel was stained with Coomassie Blue. A, purification by Ni^{2+} affinity chromatography. Lane 1, protein standards; lane 2, whole cell extract; lane 3, crude French press extract; lane 4, exclusion (unbound) fraction from the Ni^{2+} affinity column; lanes 5–8, fractions eluted successively with 0.8, 8, 40, and 80 mM imidazole buffer; lanes 9–13, fractions eluted successively with 300 mM imidazole buffer. B, cleavage of the purified fusion protein with enterokinase. The purified fusion protein was pooled and concentrated. Lane 1, protein standards; lane 2, the untreated fusion protein pool; lane 3, the pooled protein incubated for 24 h with enterokinase and subjected to gel filtration on Sephadex G-100 to remove small peptides, followed by dialysis and concentration of the peak fractions.

FIG. 3. Gel filtration of the purified fusion protein under non-denaturing conditions. A, superimposed elution profiles of a standard protein mixture and of the pool of purified fusion protein (shown in Fig. 2B, lane 2) from a Sephadex G-100 column as described under “Materials and Methods”; B, analysis of column fractions (equal volumes) by SDS-PAGE (15% acrylamide). The gel was stained with Coomassie Blue. The first lane contained the protein standard mixture, and the position of carbonic anhydrase is indicated (31kD).
fusion protein was absorbed and eluted in an almost completely
the cultures used for purification, but based on both the protein
C
 His-tagged NarJ (Fig. 7
A
). Localization of NarJ in wild-type strain RK4353. Fractions were separated by centrifugation after breakage in the French press as described under “Materials and Methods,” and equivalent volumes of each fraction were analyzed using antibodies against NarJ-His6. Lane 1, whole cell extract; lane 2, supernatant fraction; lane 3, membrane fraction.

duced levels (15–20%) compared with that of the membrane-bound complex (9). As shown in Fig. 6, the presence of NarJ appeared to stabilize the αβ complex that accumulated in the absence of the γ subunit. In this experiment, mutant MD100 was grown aerobically to midlog phase and then shifted to anaerobic conditions with the addition of nitrate to induce expression of the two subunits, and the levels of the complex were assessed in extracts of cell samples by Western blotting using anti-α subunit antibodies. In wild-type strains, this induction procedure leads to the rapid formation of nitrate reductase activity, with steady-state specific activities being reached in ~90 min (18). In MD100 (Fig. 6A), the α subunit accumulated rapidly, reaching a maximum in 60 min and then declining after 90 min. To examine the effects of NarJ on the accumulation of the complex, MD100 was transformed with plasmids that expressed either NarJ (pES203.1) or NarJ and NarI (pMV4) under the control of the tac promoter (9), and induction was carried out with the addition of IPTG to simultaneously induce these components. When both NarJ and NarI were induced with the α and β subunits (Fig. 6C), the α subunit levels increased for 60 min and then remained constant, as might be expected for the formation of the stable, membrane-bound complex. Similarly, the induction of NarJ alone with the α and β subunits led to the stabilization of the accumulated α subunits (Fig. 6B). This result suggested that NarJ may react directly with and stabilize the αβ complex prior to its interaction with the membrane-inserted γ subunit.

To look directly for an αβ-NarJ complex, we took advantage of the special characteristics of the functionally active His-tagged NarJ fusion protein. The αβ complex and His-tagged NarJ were co-induced in strain MD100(pXLJ-His6), and the resulting cells were processed and fractionated as described above for the overproduced His-tagged fusion protein. SDS gels prepared from the fractions were stained directly for protein (Fig. 7A) or blotted and separated regions of the paper stained with specific antisera against the α and β subunits (Fig. 7B) or His-tagged NarJ (Fig. 7C). In this short induction procedure, His-tagged NarJ was not overproduced to the same degree as in the cultures used for purification, but based on both the protein stain and the Western blot (Fig. 7, A and C), it is clear that the fusion protein was absorbed and eluted in an almost completely undegraded form. The α and β subunits, induced at much lower levels, were also absorbed to the Ni2+ affinity column and eluted at high imidazole concentrations in the same fractions with His-tagged NarJ (Fig. 7B). A presumed partially degraded form of the α subunit, also present in the eluted fractions, was apparently formed during the column procedure since it was not present in the crude extract (lane 3). A similar induction and fractionation procedure was carried out with strain MD100(pES203.1), which produced both NarJ and the αβ complex; in this case, most of the NarJ protein and α and β subunits were not absorbed to the column, and the remainder of each was washed off at low concentrations of imidazole (data not shown). These results demonstrate that overproduced His-tagged NarJ formed a complex with the αβ complex that was sufficiently stable to be purified by binding to and elution from the Ni2+ affinity resin.
DISCUSSION

The narJ gene is the third of four contiguous open reading frame in the narGHJI operon, and its start and stop codons overlap by 1 base pair each of the adjacent open reading frames (19). In general, this type of overlapping structure has been thought to lead to translational coupling and the formation of similar amounts of each of the operon products (20). This did not seem to be the case for the NarJ protein; less than stoichiometric levels of NarJ accumulated with the T7 expression even though NarJ appeared to be just as stable as the other operon proteins. Furthermore, only low levels of NarJ could be accumulated when fragments containing the narJ gene were expressed from multicopy plasmids under the control of the tac promoter. These results suggested that, relative to the other operon products, the expression of NarJ is restricted at the level of translation and that less than stoichiometric levels of NarJ are required for the assembly of nitrate reductase.

Based on its properties, the NarJ protein would appear to fit the formal definition of a molecular chaperone. It is a protein that is required for the assembly of the nitrate reductase complex on the membrane; it forms a complex with the α and β subunits prior to their assembly with the membrane-integrated γ subunit; and after assembly of the αβγ complex on the membrane, NarJ remains in the cytosol.

In contrast to general molecular chaperones (21), NarJ would have to be defined as a system-specific chaperone since it appears to be required only for the biogenesis of nitrate reductase. As a component of the narGHJI operon, it is expressed only when nitrate reductase formation is induced, and its deletion appears to have little effect on the growth and physiology of the affected cells except that which is attributable to the absence of functional nitrate reductase. Genes related to narJ have been identified in other operons that encode membrane-bound nitrate reductase complexes (22–24), but data base searches have not revealed significant homologies to any other known genes that might provide clues concerning the role of NarJ or its homologues in the biogenesis of nitrate reductase.

What possible role might NarJ play in the assembly of the nitrate reductase complex? Previous studies have established that a cytoplasmic αβ complex, accumulated in mutants that produce no membrane-integrated γ subunit, is partially active when NarJ is present, but is inactive in mutants that lack NarJ as well (9, 10). When purified, the inactive form contained a full complement of cofactors (10) and appeared to be readily degraded by endogenous (9) and exogenous (10) proteases. The αβ complex accumulated in the presence of NarJ catalyzed the reduced methyl viologen-nitrate reductase reaction at 15–20% the rate catalyzed by the membrane-bound complex (9), and it appeared to be less sensitive to endogenous protease degradation than the inactive αβ complex accumulated in the absence of NarJ. Furthermore, Palmer et al. (25) have shown recently that, during the in vitro incorporation of molybdopterin cofactor, NarJ is one of the components required to form active nitrate reductase. Together, these observations suggest that NarJ facilitates a maturation or refolding of the inactive αβ complex to an active, protease-resistant complex.

Since the inactive αβ complex does not form a complex with the membrane-bound γ subunit in the absence of NarJ (9, 10), it seems likely that either the active “matured” form of αβ or the form bound to NarJ is required for interaction with the membrane-integrated γ subunit and assembly of the active αβγ complex. A detailed characterization of the structure and activity of the αβγNarJ complex should provide more definitive information about the steps involved in assembly of nitrate reductase and the role of NarJ in this process.

Putative system-specific or “private” chaperones have been identified for several other unrelated systems, including extra-cellular lipase formation (limA) in Pseudomonas cepacia (26), urease formation (ureD) in Klebsiella aerogenes (27), pilus formation (papD) in E. coli, (28), and F1-ATPase assembly (ATP11, ATP12) in Saccharomyces cerevisiae (29). In each case, the identified component, like NarJ, is required for assembly or processing, but is not found associated with or required for activity or function of the final product. None of these systems-specific chaperones have structural features or apparent activities in common that might imply general functional homologies. It is possible that, in each case, a specific problem in biogenesis can be solved only by the participation of a specific protein factor. It is also possible that some general function is shared by these factors, such as protection or formation of otherwise reactive intermediates in the assembly process or facilitation of an interaction of intermediates with the general molecular chaperones of the cell to complete folding steps that are critical to the assembly process.

Addendum—After completion of this manuscript, we learned that Giordano et al. have purified NarJ by a similar approach and have demonstrated that NarJ interacts with the αβ complex in vitro, resulting in the formation of active enzyme.

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