SHORT COMMUNICATIONS

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Immunochemical studies on the interrelationships between sulfite reductase and other NADPH-linked reductase activities of *Escherichia coli* and *Salmonella typhimurium*

In previous investigations[1-5] considerable evidence has been adduced to show that the NADPH-linked sulfite reductase (H_2S:NADP oxidoreductase, EC 1.8.1.2) of *Escherichia coli* and *Salmonella typhimurium* is also capable of catalyzing the reduction of hydroxylamine and nitrite. The 3 reductase activities, as measured in cell-free extracts, have been shown to be related to each other by a constant numerical ratio throughout different stages of purification, to be coordinately enhanced or repressed in cells grown in the presence of serine or cysteine, respectively, and to be competitively inhibited each by the alternate substrates as well as by cyanide and arsenite[1-7]. Furthermore, genetic analysis has revealed that the formation of the NADPH-linked sulfite reductase with triple substrate specificity is governed by 6 cistrons, 4 of which are also instrumental in controlling the ability to catalyze the NADPH-dependent reduction of FAD and cytochrome *c* which is co-repressed by cysteine[7,8]. Thus, mutants blocked in cistrons Ba, Bb, Bc and J were unable to grow on sulfite as the sole source of sulfur and were deficient in all the above-mentioned reductase activities. In contrast, mutants blocked in cistrons G or I were incapable of reducing sulfite, hydroxylamine and nitrite but possessed an unimpaired capacity for reduction of FAD and cytochrome *c*. It was inferred from the latter findings that the sulfite reductase system involves at least 2 separate components, one of which is shared by the cytochrome *c* reductase[7]. The essential validity of this interpretation seems to be borne out and corroborated by the results of the present study.

The bacterial strains of *E. coli* B and *S. typhimurium* were grown with continuous shaking at 37° in the standard medium of the following composition: 0.2% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, 0.3% $Na_2HPO_4 \cdot 12H_2O$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.001% $CaCl_2 \cdot 2H_2O$, 0.2% glucose and 0.3% DL-serine; final pH 7.4. The mutant strain of *E. coli* B lacking sulfite reductase was grown in the same medium but supplemented with 1 mM Na_2S. For repression of sulfite reductase formation in *E. coli* B, DL-serine in the medium was replaced by 0.3 mM L-cysteine.

The cells harvested in the late logarithmic phase were washed with 0.9% NaCl solution, suspended in cold 50 mM potassium phosphate buffer (pH 7.4) to a concentration of 20% (wet wt.) and 10-ml portions of the suspension were mixed with an equal weight of glass beads (*d*, 0.12 mm). All the subsequent operations were carried out in the cold. Disruption of the cells was effected by shaking the mixture for 20 sec in a refrigerated Nossal oscillator and the resultant cell-free extract was separated by centrifugation at 20 000 × *g*. Following dialysis against 5 mM phosphate buffer (pH 7.4), the bulk of the nucleic acids was removed by precipitation with streptomycin sulfate at a final concentration of 0.9%, and the supernatant was subjected to $(NH_4)_2SO_4$ fractionation. The fraction precipitated between 35% and 50% $(NH_4)_2SO_4$ saturation (fraction I) was dialyzed and dissolved in 0.01 M phosphate buffer (pH

7.5); it showed an about 5-fold increase in the specific activity of sulfite reductase over that of the crude extract. The fraction I solution was brought to 10% $(NH_4)_2SO_4$ saturation and was further purified by treatment with acetone at −12°. The precipitate formed on raising the acetone concentration from 40% to 50% (v/v) (fraction II) was collected. The specific activity of sulfite reductase at this stage of purification was about 10-fold higher than that of the crude extract. Fraction II dissolved in 0.05 M phosphate buffer (pH 8) was adsorbed on calcium phosphate gel (1.7 mg dry wt./mg protein) and was then eluted with 0.2 M potassium phosphate buffer at pH 7 (fraction III). The overall procedure resulted in a more than 100-fold purification of the sulfite reductase. The enzymic assay procedures used were essentially similar to those described by KAMIN *et al.*[5].

The antisera were produced in rabbits by 6 suitably spaced injections of the partially purified extracts (fraction I) from the different bacterial strains supplemented with Freund's adjuvant (Difco).

When a purified enzyme preparation (fraction III) of *E. coli* B or *S. typhimurium* was mixed with homologous antiserum, an inhibition of the different reductase activities took place. At a given level of antiserum, the extent of reductase inhibition proved to be virtually the same with either sulfite, hydroxylamine or nitrite used as electron acceptors. Contrasting with this parallel response, the inhibitory effect of the antiserum on the ability to reduce FAD and cytochrome *c* was much less pronounced. Thus, an amount of antiserum which gave rise to a 90% inhibition of the sulfite reductase, produced only a 40% drop in the rates of FAD and cytochrome *c* reduction catalyzed by the same cell-free extracts. The specificity of the antiserum action was attested to by the observation that the NADPH-linked reductases showed no inhibition with normal rabbit serum or antiserum produced against an extract from *E. coli* B cells in which the formation of sulfite reductase and cytochrome *c* reductase activities had been completely repressed by growth in the presence of cysteine[1].

Immunoelectrophoretic analysis of a partially purified preparation of sulfite reductase (fraction II) from *E. coli* B with homologous antiserum revealed 4–5 distinct precipitation arcs (Fig. 1A), whereas the more highly purified preparation (fraction III) yielded with the same serum 3 arcs only (Fig. 3B). At least two of the arcs (including the one marked by an arrow in Fig. 1A) were missing, when the same enzyme preparation was tested with an antiserum produced against an extract from

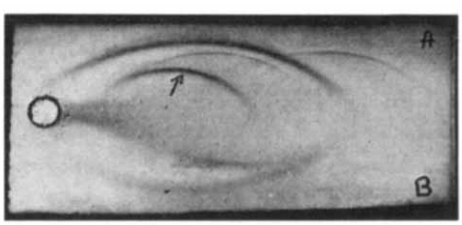

Fig. 1. Immunoelectrophoresis of a partially purified sulfite reductase preparation of *E. coli* B. The enzyme preparation (fraction II) was placed in the central (antigen) well. Upper trough (A): homologous antiserum produced against fraction I of *E. coli* B grown in the standard medium. Lower trough (B): antiserum against fraction II of the same strain, but cells grown in the presence of cysteine. The electrophoresis was carried out in agar gel buffered with barbital at pH 8.6 using the conventional microprocedure[9].

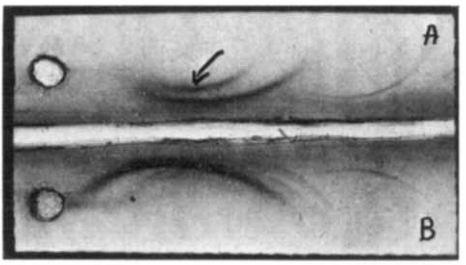

Fig. 2. Immunoelectrophoretic analysis of purified extracts (fraction II) of *S. typhimurium* and a sulfide-requiring mutant of *E. coli* B lacking sulfite reductase activity. Top well (A): *S. typhimurium* extract. Bottom well (B): *E. coli*-mutant extract. The central trough contained antiserum produced against an extract (fraction I) of *E. coli* B wild type. Otherwise conditions were as in Fig. 1.

cysteine-repressed cultures of *E. coli* B (Fig. 1B). The characteristic precipitation arc failed to appear also when antiserum produced against the *E. coli* B wild-type strain was allowed to interact with cell-free extracts from a sulfide-requiring mutant strain of *E. coli* B which was unable to utilize sulfate or sulfite for the biosynthesis of cysteine (see Fig. 2B). The mutant-strain extracts contained negligibly low titers of sulfite reductase but showed normal levels of cytochrome *c* reductase and FAD reductase. On the other hand, purified extracts from a revertant derived from the mutant strain which had regained normal sulfite reductase activity, yielded an immunoelectrophoretic pattern which was indistinguishable from that exhibited by the congeneric wild-type strain preparations. Furthermore, as shown in Fig. 2, the immunoelectrophoretic pattern of a sulfite reductase preparation (fraction II) from *S. typhimurium* with antiserum against *E. coli* B, shared at least 4 precipitation arcs with the corresponding *E. coli* B preparation. The cross-antigenicity of the extracts derived from the two kindred organisms was also reflected in the mutual inhibition of their sulfite reductases produced by the respective antisera.

The existence of a recognizable sulfite reductase specific arc was demonstrated in a more direct manner by using a "specific" antiserum (produced against the wild-type *E. coli* B extract) from which antibodies, other than those directed against the sulfite reductase antigen, had been eliminated by precipitation with an extract derived

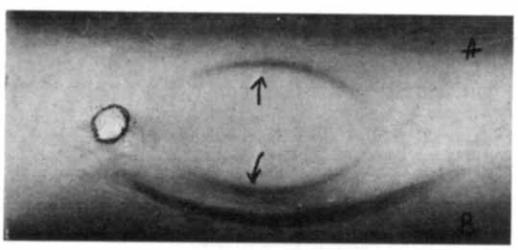

Fig. 3. Immunoelectrophoretic patterns of a purified sulfite reductase preparation of *E. coli* B obtained with untreated and "specific" antisera. Central well: enzyme preparation (fraction III). Upper trough (A): antiserum produced against the wild-type *E. coli* B extract, rendered specific for sulfite reductase by prior precipitation with an extract (fraction I) from the sulfiderequiring mutant devoid of sulfite reductase. Lower trough (B): antiserum produced against the wild-type *E. coli* B extract (fraction I), untreated.

from the mutant lacking sulfite reductase activity or from a cysteine-repressed culture of the wild-type strain. As shown in Fig. 3A, the serum so treated reacted with the purified enzyme preparation (fraction III) of *E. coli* B to yield a single immunoprecipitation arc which appeared to be identical to one of the 3 arcs (proximal to the antigen well) formed by the untreated antiserum with the same enzyme preparation (Fig. 3B). No precipitation line was obtained when the sulfite reductase specific antiserum was tested against an extract from cysteine-repressed *E. coli* B cells with no detectable sulfite reductase activity, or when the antiserum employed had been deprived of the antibodies by precipitation with a purified preparation of sulfite reductase.

It may be concluded, therefore, that the present data provide evidence for the existence of an antigenic entity, specifically associated with the NADPH-linked reductase mediating the reduction of sulfite, hydroxylamine and nitrite and apparently unrelated to the cytochrome *c* and FAD reductase activities. The question, however, of whether these genetically interrelated activities are inherent in separate molecular species, or represent different and discrete moieties (subunits) of a composite protein molecule cannot yet be answered conclusively.

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