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Immunological crossreactivity of eukaryotic C_1-tetrahydrofolate synthase and prokaryotic 10-formyltetrahydrofolate synthetase

(immunoblot/antigen crossreaction/electrophoretic transfer blot/folate coenzymes)

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ABSTRACT **Antiserum to yeast C_1-tetrahydrofolate (C_1-H_4folate) synthase reacts with other eukaryotic C_1-H_4folate synthases and prokaryotic 10-formyltetrahydrofolate (10-CHO-H_4folate) synthetases [formate:tetrahydrofolate ligase (ADP-forming), EC 6.3.4.3] even though these enzymes vary in subunit size and function and probably vary widely in sequence. The comigration of the purified enzymes with the immunoreactive material establishes the specificity of the reaction for C_1-H_4folate synthase proteins. Reciprocal crossreaction of the antibody to *Clostridium acidi-urici* 10-CHO-H_4folate synthetase with the eukaryotic proteins indicates that such broad cross-species reactions are not specific to the antisera elicited in response to the yeast C_1-H_4folate synthase. These specific crossreactions among divergent species have been observed only on an electrophoretic transfer blot of a denaturing polyacrylamide gel. These observations may have been possible because of the sensitivity and specificity of the technique, which differ from more conventional immunochemical methods.**

Crossreaction between the same protein from different species has been observed for many conserved proteins, for example cytochrome *c* or lysozyme. However, the proteins that crossreact have nearly identical subunit molecular weights and extremely similar structures, even at the tertiary level. We report an example of crossreaction between proteins from eukaryotes and prokaryotes that have related enzymatic functions but that vary in size, function, and sequence.

N^{10}-Formyltetrahydrofolate (10-CHO-H_4folate) synthetases [formate:tetrahydrofolate ligase (ADP-forming), EC 6.3.4.3] serve diverse metabolic roles and are organized onto proteins differently in various organisms. We have been studying *Saccharomyces cerevisiae* C_1-tetrahydrofolate (C_1-H_4folate) synthase, a trifunctional enzyme that has 10-CHO-H_4folate synthetase (EC 6.3.4.3), N^5,N^{10}-methenyltetrahydrofolate (5,10-CH^+-H_4folate) cyclohydrolase (EC 3.5.4.9), and N^5,N^{10}-methylenetetrahydrofolate (5,10-CH_2-H_4folate) dehydrogenase (EC 1.5.1.5) activities on one protein composed of two identical polypeptides (1). As part of this investigation, we are trying to compare this trifunctional protein to the trifunctional C_1-H_4folate synthases found in other eukaryotes, some of which have been purified (1–5), and to the monofunctional 10-CHO-H_4folate synthetases found in two purine-fermenting *Clostridia*, *C. acidi-urici* and *C. cylindrosporum* (6). Electrophoretic transfer blotting reveals a relationship between the proteins not detected by microcomplement fixation or Ouchterlony precipitation (7).

Aside from yielding information about the relatedness of C_1-H_4folate synthase and 10-CHO-H_4folate synthetase proteins, the reaction of the anti-C_1-H_4folate synthase antibody with the widely variant proteins of different molecular weights is a graphic example of wide crossreactivity between eukaryotic and prokaryotic proteins of similar function but dissimilar size and structure.

MATERIALS AND METHODS

Enzyme Purification. Yeast C_1-H_4folate synthase, coded for by the *ADE3* locus, was purified to homogeneity, judged by $NaDodSO_4$ gel electrophoresis, from yeast strain M1614C, which is wild-type with respect to the *ADE3* locus (see Fig. 1*A*, lane 3). The procedure was an unpublished affinity method that yielded enzyme apparently identical to that purified by other procedures in our laboratory (1, 8). Assays for the activities of the C_1-H_4folate synthase enzymes were essentially as described in the preceding references. 10-CHO-H_4folate synthetases from *C. acidi-urici* and *C. cylindrosporum* were purified in this laboratory (9). Purified rabbit (4), chicken (3), and pig liver (5) C_1-H_4folate synthases were the gifts of L. Schirch, S. J. Benkovic, and R. MacKenzie, respectively.

Antibody Preparation. Antibody to yeast C_1-H_4folate synthase used in these experiments was produced by injecting 150 μg of purified yeast (strain M1614C) C_1-H_4folate synthase suspended in 2 ml of complete Freund's adjuvant (Bacto) at two sites in the back muscles of 3-kg female New Zealand White rabbits. After 6 wk, a booster of 75 μg of C_1-H_4folate synthase in 1 ml of complete adjuvant was injected in the same sites. Antisera used in this study were collected 13 wk after the initial injection. The blood was clotted and centrifuged, and the amber supernatant fluid was frozen at −20°C. Three rabbits were used in this study; all gave nearly identical titers: 1 μl of serum precipitated 1 μg of purified yeast C_1-H_4folate synthase. The antisera to *C. acidi-urici* 10-CHO-H_4folate synthetase were prepared by A. Champion in this laboratory (7).

Preparation of Crude Extracts. All extracts were made in buffer A (25 mM Tris·H_2SO_4/10 mM KCl/10 mM 2-mercaptoethanol/1 mM phenylmethylsulfonyl fluoride). All tissues or cells had been stored at −80°C prior to use. Chicken livers, rabbit livers, spinach, and *Drosophila melanogaster* flies were homogenized in a Polytron homogenizer (Brinkmann). *C. cylindrosporum* and *C. acidi-urici* were grown as described (9). *Escherichia coli* (strain HB101) was grown in LB broth. The suspended bacterial cells were sonicated for three bursts of 20 seconds in a Branson sonicator. Yeast extracts were prepared from a strain that was wild-type for the *ADE3* locus, M1614C, and a strain with a deletion in this locus, *ade*3-5281. The yeast cells were treated for 1 min in a Bead-Beater (Biospec Products, Bartlesville, OK). After homogenization, all extracts were cleared by centrifugation at 20,000 rpm (40,000 × *g*) for 20 min in a Sorvall SE-12 rotor and stored at −80°C. Protein concentrations of the extracts were determined by the method of Brad-

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Abbreviations: H_4folate, tetrahydrofolate; 10-CHO-H_4folate, N^{10}-formyltetrahydrofolate; 5,10-CH^+-H_4folate, N^5,N^{10}-methenyltetrahydrofolate; 5,10-CH_2-H_4folate, N^5,N^{10}-methylenetetrahydrofolate.

ford (10) with Bio-Rad's Coomassie blue reagent. Bovine serum albumin (Armour Pharmaceuticals, Phoenix, AZ) was used as a standard.

Immunoblotting Technique. All $NaDodSO_4$/polyacrylamide gels were run essentially as described by Laemmli (11). Proteins were transferred to nitrocellulose as described by Burnette (12) and immunostained with peroxidase (13). Specifically, after transfer overnight, the gels were soaked for 1 hr at 42°C in Tris-buffered saline/3% bovine serum albumin/10% heat-inactivated calf serum. This solution was replaced with a dilution of antiserum (in the same buffer) and incubated at room temperature for 1 hr with agitation. A 1:500 dilution of antibodies against yeast C_1-H_4folate synthase or a 1:100 dilution of antibodies against *C. acidi-urici* 10-CHO-H_4folate synthetase were used as the specific antisera, as indicated. The blots were washed five times (2 min each) with Tris-buffered saline, then incubated for 1 hr with a 1:3,000 dilution of goat anti-rabbit IgG coupled to horseradish peroxidase (Bio-Rad), and rinsed with Tris-buffered saline as before. The stain was visualized with 4-chloronaphthol as described by Hawkes *et al.* (14). As little as 50 pg of yeast C_1-H_4folate synthase can be detected by this assay.

Affinity Purification of Antisera. C_1-H_4folate synthase agarose was prepared by coupling 1 mg of pure yeast C_1-H_4folate synthase to 0.75 g of Bio-Rad Affi-Gel 10 with conditions as described by the manufacturer. *C. acidi-urici* 10-CHO-H_4folate synthetase agarose was prepared by the same method. These resins were used to fractionate crude antiserum as described by Shapiro *et al.* (15). High-affinity antibodies were eluted with 4.5 M $MgCl_2$. All fractions were dialyzed against Tris-buffered saline immediately after elution.

Immunoprecipitation of 10-CHO-H_4folate Synthetase and 5,10-CH_2-H_4folate Dehydrogenase Activities. Immunoprecipitations with *Staphylococcus aureus* cells were as described by Kessler (16). The protein extracts were the same as were used for the immunoblot experiment, but they were used at a protein concentration of 3 mg/ml, except for the *C. acidi-urici* and *C. cylindrosporum* extracts, which were 30 μg/ml. The mixtures contained 10 μl of antiserum to yeast C_1-H_4folate synthase per 100 μl of extract and were incubated 3 hr on ice before the addition of immunoprecipitation buffer and 10 μl of 25% *S. aureus* cells. Incubation was continued for 1 hr on ice, the cells and their bound immunoglobulin were centrifuged from solution, and the supernatant solutions were removed for assay.

RESULTS

Immunoblot Experiment. Crude protein extracts of widely divergent organisms contained proteins that crossreacted with rabbit antiserum raised in response to yeast C_1-H_4folate synthase. This crossreaction was apparent in the antibody-stained electrophoretic transfer blot (Fig. 1*B*). When compared to the Coomassie blue stain of the proteins present in the cell extracts (Fig. 1*A*), the antibody reacted with a limited set of proteins. These proteins varied in molecular weight from 50,000–160,000 in the different extracts. These reactions depended upon the anti-C_1-H_4folate synthase antiserum; a duplicate blot probed with serum from the same rabbit prior to immunization gave no detectable stain. Also, these crossreactions depended upon the particular antiserum used; only one of three rabbits yielded antiserum with such broad crossreactivity, although all three antisera reacted with yeast C_1-H_4folate synthase.

Specificity of the Antiserum. The purity of the antigen used to elicit polyclonal antisera must be high to insure the specificity of the sera. Although we used a highly-purified preparation of yeast C_1-H_4folate synthase as an immunogen, the cross-species reactions that we observed could be due to minor, highly immunogenic contaminants of our yeast C_1-H_4folate synthase preparation, which could have caused a nonspecific crossreaction. However, the comigration of the purified yeast, chicken, rabbit, and *C. acidi-urici* 10-CHO-H_4folate synthetase proteins with their respective antibody-staining bands (compare lanes 3, 5, 7, and 9 in Fig. 1 *A* and *B*) established that this antiserum detects C_1-H_4folate synthase-related proteins, even in divergent organisms. Although the other (minor) bands in these extracts may represent reactions with unrelated proteins, the essential point is that antibody to yeast C_1-H_4folate synthase reacts with all characterized C_1-H_4folate synthase-related proteins available.

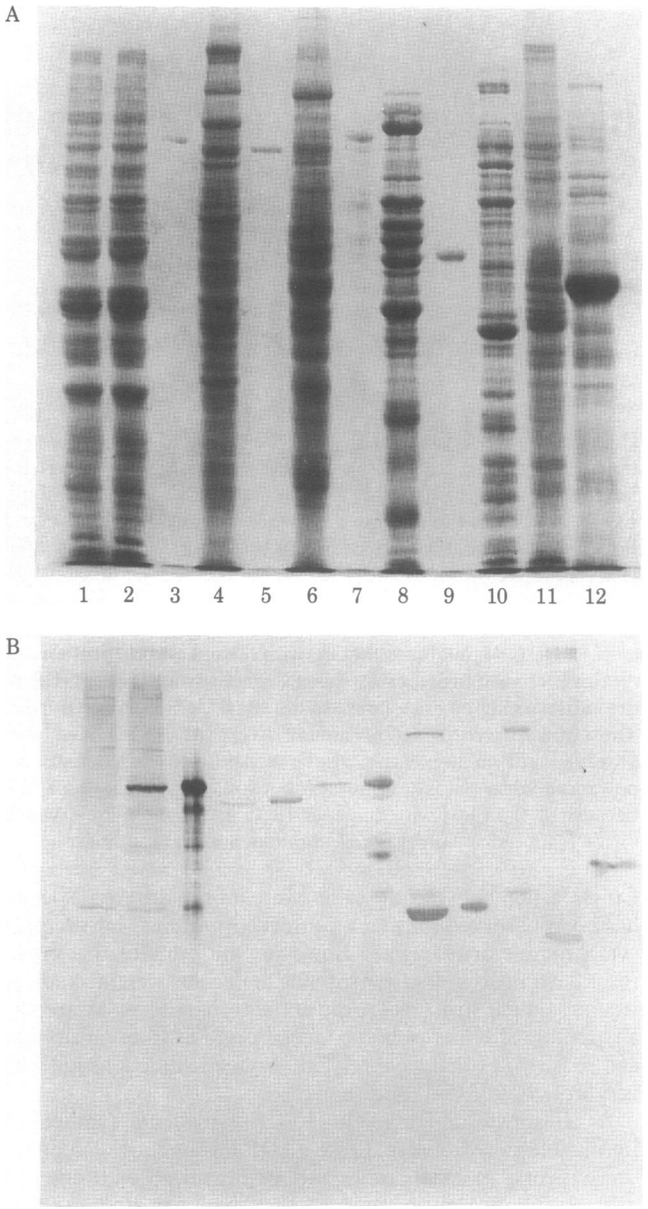

FIG. 1. (*A*) Coomassie blue stain of all proteins present in the extracts and purified fractions. (*B*) Nitrocellulose replica of the gel in *A*, stained with antibodies to yeast C_1-H_4folate synthase as described. Lanes on both gels contained the following proteins: 1, yeast extract from *ADE3* deletion strain (50 μg); 2, M1614C yeast extract (50 μg); 3, yeast C_1-H_4folate synthase (0.2 μg); 4, chicken liver extract (50 μg); 5, chicken C_1-H_4folate synthase (0.5 μg); 6, rabbit liver extract (50 μg); 7, rabbit C_1-H_4folate synthase (1 μg); 8, *C. acidi-urici* extract (50 μg); 9, *C. acidi-urici* 10-CHO-H_4folate synthetase (1 μg); 10, *E. coli* extract (50 μg); 11, *D. melanogaster* extract (50 μg); 12, spinach extract (50 μg).

The reactions of anti-C_1-H_4folate synthase with each of the extracts were more detailed than the previous discussion suggests. Yeast crude extract (Fig. 1*B*, lane 2) contained one major antibody-staining band that comigrated with the pure C_1-H_4folate synthase from that organism (lane 3). This band was not present in extracts of a strain of yeast that has a large deletion in the gene coding for the protein (lane 1). Many of the bands below the major one in lane 2 appeared to be proteolysis products of the intact enzyme because they were absent in the deletion strain extract. We have not identified the other bands that are present in both extracts, though we have preliminary evidence that a M_r 60,000 protein that was stained by antibody may be a mitochondrial form of 10-CHO-H_4folate synthetase. The antiserum reacted with the purified chicken (lane 5) and rabbit (lane 7) C_1-H_4folate synthases and with the monofunctional *C. acidi-urici* 10-CHO-H_4folate synthetase (lane 9). One additional band stained in the *C. acidi-urici* extract (lane 8), but this band was not present in the purified *C. acidi-urici* 10-CHO-H_4folate synthetase. Bands also were observed in the *E. coli*, *D. melanogaster*, and spinach extracts (lanes 10, 11, and 12). The molecular weights of these bands and the reported molecular weights of C_1-H_4folate synthase proteins from these organisms (if known) are compared in Table 1. The antiserum also stained the pig and sheep C_1-H_4folate synthases in data not shown. One reaction we have not explained is that the antiserum stained a band larger than that of 10-CHO-H_4folate synthetase, rather than 10-CHO-H_4folate synthetase, in the *C. cylindrosporum* extract.

Reciprocal Crossreaction with Antisera to *C. acidi-urici* 10-CHO-H_4folate Synthetase. An experiment similar to that in Fig. 1, with the substitution of a pooled antiserum to *C. acidi-urici* 10-CHO-H_4folate synthetase for the antiserum to yeast C_1-H_4folate synthase, yielded a similar reaction pattern (Fig. 2). The antiserum to *C. acidi-urici* 10-CHO-H_4folate synthetase reacted with *C. acidi-urici* 10-CHO-H_4folate synthetase, as expected (lane 9). In addition, it reacted with the *C. cylindrosporum* 10-CHO-H_4folate synthetase (lane 10), which comigrated with the *C. acidi-urici* 10-CHO-H_4folate synthetase and the purified yeast, chicken, and rabbit C_1-H_4folate synthases (lanes 3, 5, and 7). The antiserum to *C. acidi-urici* 10-CHO-H_4folate synthetase also reacted with a high molecular weight protein in the *C. acidi-urici* extract (lane 8) that was not present in the purified *C. acidi-urici* 10-CHO-H_4folate synthetase, which seems to be the same as that detected by the antiserum to yeast C_1-H_4folate synthase. The antiserum also reacted with a M_r 60,000 protein in the chicken extract (lane 4). The antiserum to *C. acidi-urici* 10-CHO-H_4folate synthetase reacted with many bands in the *E. coli* extract (lane 11) but with only a few bands in the *Drosophila* and spinach extracts (lanes 12 and 13). Some of these bands seemed to be the same as those with which the antiserum to yeast C_1-H_4folate synthase reacted, though some were different. In short, the antiserum to *C. acidi-urici* 10-CHO-H_4folate synthetase reacted with all of the purified 10-CHO-H_4folate synthetase and C_1-H_4folate synthase proteins as well as with most of the same proteins with which the antiserum to yeast C_1-H_4folate synthase reacted.

Table 1. Comparison of immunoreactive proteins and reported molecular weights for C_1-H_4folate synthase proteins

Organism	$M_r \times 10^{-3}$ Immunoreactive band	$M_r \times 10^{-3}$ Reported value*	Ref.
Yeast	160		
	126		
	104.5	104.5	(1)
Chicken	99	90–97	(5)
Rabbit	107	215 (native)	(4)
C. acidi-urici	136		
	60	60	(3)
E. coli	138	30.6, 28.2, 24.5	(17)
	65	21.2, 16.5	
D. melanogaster	53		
Spinach	75		

Molecular weights were estimated from the $NaDodSO_4$/polyacrylamide gel in Fig. 1 by assuming the reported molecular weights of the yeast C_1-H_4folate synthase and *C. acidi-urici* 10-CHO-H_4folate synthetase as given in the table. The immunoreactive bands are the major bands in each extract that react with antibody to yeast C_1-H_4folate synthase.

*For C_1-H_4folate synthase or 10-CHO-H_4folate synthetase.

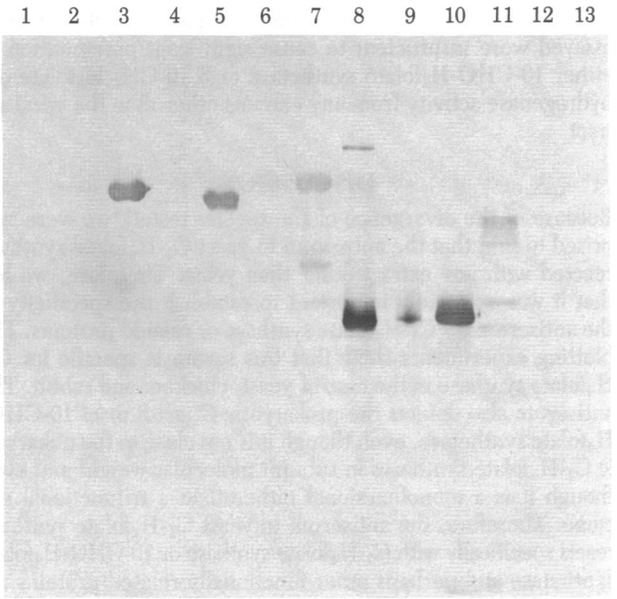

FIG. 2. Nitrocellulose replica of a gel similar to that in Fig. 1 but stained with antiserum to *C. acidi-urici* 10-CHO-H_4folate synthetase. Lanes: 1–7, same as in Fig. 1; 8, *C. acidi-urici* extract (5 μg); 9, *C. acidi-urici* 10-CHO-H_4folate synthetase (0.1 μg); 10, *C. cylindrosporum* extract (12 μg); 11–13, same as lanes 10–12 in Fig. 1.

Affinity Purification of the Antiserum. Affinity purification of the antiserum to yeast C_1-H_4folate synthase resulted in two fractions with antibody activity. The low-affinity antibodies were eluted under mild conditions from a C_1-H_4folate synthase agarose column, whereas the higher-affinity pool required chaotropic agents to effect elution. Substantial loss of antibody activity occurred with the column, probably due to the failure either to elute all of the applied antibody or to renature the antibody that was eluted with the chaotropic agent. However, high-affinity antibody clearly crossreacted with the spinach protein and with the *C. acidi-urici* and chicken proteins as well. The low-affinity pool mirrored the response of the crude antiserum. We would like to note in particular that the low-affinity antibodies reacted with the *C. acidi-urici* 10-CHO-H_4folate synthetase, indicating that even low-affinity antibodies react specifically with 10-CHO-H_4folate synthetase.

Affinity purification of the antiserum to *C. acidi-urici* 10-CHO-H_4folate synthetase resulted in a high-affinity pool that reacted only with the *C. acidi-urici* and *C. cylindrosporum* 10-CHO-H_4folate synthetases. The reactivity of the low-affinity pool was not examined in this case.

Immunoprecipitation. Yeast C_1-H_4folate synthase can be precipitated from solution by the antiserum to C_1-H_4folate synthase and *S. aureus* cells. Conditions sufficient to precipitate a

200-fold excess of yeast C_1-H_4folate synthase over the amounts assayed were insufficient to cause significant precipitation of either 10-CHO-H_4folate synthetase or 5,10-CH_2-H_4folate dehydrogenase activity from any extracts other than the yeast extract.

DISCUSSION

Because of the divergence of the species tested, we were surprised to find that the antiserum to yeast C_1-H_4folate synthase reacted with any extract other than yeast. Therefore, we felt that it was extremely important to establish the specificity of the antiserum for C_1-H_4folate synthase or related proteins. The blotting experiments show that this serum is specific for C_1-H_4folate synthase in the case of yeast, chicken, and rabbit. The antiserum also detects the prokaryotic *C. acidi-urici* 10-CHO-H_4folate synthetase, even though it is not close to the eukaryotic C_1-H_4folate synthases in subunit molecular weight and even though it is a monofunctional rather than a trifunctional enzyme. Therefore, the antiserum to yeast C_1-H_4folate synthase reacts specifically with C_1-H_4folate synthase or 10-CHO-H_4folate synthetase and perhaps other functionally related proteins.

The reciprocal crossreactions between the yeast and *C. acidi-urici* antisera and C_1-H_4folate synthase and 10-CHO-H_4folate synthetase suggest even more strongly that the crossreactions are not artifacts peculiar to the anti-yeast antiserum but a general feature of the C_1-H_4folate synthases. Although the two antisera do not react with exactly the same set of proteins, the overlap in the sets that are detected includes most of the major reactive species.

The affinity fractionation of the antisera indicated that the members of the population that bound to their affinity adsorbents most tightly showed the narrowest spectra of crossreaction. Apparently, the broadest cross-species reactivity is found among the lowest-affinity antibodies. However, quite divergent proteins react with the high-affinity antibodies, and even the lower-affinity interactions are specific.

To try to clarify the relationship between the antibody-staining bands and the C_1-H_4folate synthase activities, particularly in those extracts such as *E. coli*, spinach, and *Drosophila* for which we had no purified enzymes available, we undertook immunoprecipitation of the C_1-H_4folate synthase activities from the extracts. However, we were unable to precipitate the activities from the extracts except in the case of yeast. We can suggest two explanations for the inability to precipitate the activities. First, it seems likely that only a small fraction of the antibodies in the polyclonal antiserum crossreact with the other enzymes. If less than 1% of the antibodies were crossreactive, we would have difficulty observing immunoprecipitation. A second possible explanation is that the antibodies recognize a structure present only after the proteins have been denatured and transferred to the nitrocellulose. The crossreaction of antibodies to denatured hen egg white lysozyme with denatured human leukemia lysozyme, even though the native proteins do not crossreact, is one example of the broadening of specificity by denaturation (18). However, the antigen which we used to inoculate the rabbits, unlike the lysozyme, had not been treated with denaturants. At this point, we cannot distinguish these possibilities.

To interpret the immunoblot crossreactivity, it is important to compare the immunoblot technique to other immunochemical measures of protein relatedness. For example, precipitin tests and microcomplement fixation, which rely on multivalent antibody–antigen interactions, do not detect similarity in proteins that differ more than 40% in sequence (19). Such tests applied to Clostridial 10-CHO-H_4folate synthetases indicated no homology (7). Tests which detect monovalent interactions, such as immunoprecipitation, immunoinactivation, or immunoblotting can indicate a relationship between more distantly related proteins than do tests that require multivalent interactions. For example, antibodies to one dehydrogenase inhibit several eukaryotic dehydrogenases, apparently by binding at a conserved NADPH-binding site (20). Antibodies that mimic the sweet taste receptor bind the protein that elicited them, thaumatin, and other sweet substances that may have similar structures but are quite different substances, such as sucrose or saccharin (21).

We conclude that the antibody to yeast C_1-H_4folate synthase reacts with a structure or sequence common to 10-CHO-H_4folate synthetases from widely divergent organisms. Because the proteins that react in these immunoblots are from divergent organisms, have different subunit molecular weights, and, in some cases, have been shown not to be highly homologous, we interpret the crossreactions we observe as due to small, specific regions of the proteins that share structure or sequence features. We suspect that these regions may be H_4folate binding sites or domains, analogous to the nucleotide binding domains recognized by Rossmann *et al.* (22). These sites are present on proteins that have similar enzymatic functions, even though they have evolved otherwise different structures. The electrophoretic transfer blot technique allows one to visualize these specific crossreactions by using polyclonal antisera that do not show relationships between the proteins by other immunochemical techniques.

Aside from considering the immunological relationship between C_1-H_4folate synthase and related proteins, these experiments were an opportunity to directly compare the purified yeast, chicken, rabbit, and pig C_1-H_4folate synthases. The similar subunit molecular weights, native dimeric structure, and immunological crossreaction between these enzymes suggest structural similarity. However, the proteins may differ functionally. For example, the yeast C_1-H_4folate synthase has far more 10-CHO-H_4folate synthetase activity relative to the 5,10-CH_2-H_4folate dehydrogenase (8) than does the rabbit C_1-H_4folate synthase (4). As well as confirming the reported molecular weights of the eukaryotic C_1-H_4folate synthases, the immunoblots may give information about H_4folate enzymes in other organisms. For example, the band we observed in the *C. acidi-urici* extract aside from 10-CHO-H_4folate synthetase could be a dehydrogenase or cyclohydrolase, although the reported molecular weight of the dehydrogenase, 70,000 (23), is lower than that of the immunoreactive band. The bands in the *E. coli* extract do not correspond to any of the molecular weights reported for the dehydrogenase/cyclohydrolase with the unusual subunit composition purified by Dev and Harvey (17). We, like they, are unable to demonstrate 10-CHO-H_4folate synthetase in *E. coli* extracts, so we presume that the bands we observe cannot be due to this enzyme but may be related to the other activities of C_1-H_4folate synthase. We have no information concerning the subunit size of the *Drosophila* or spinach C_1-H_4folate synthases, though we know that both sources do contain each of the C_1-H_4folate synthase enzymes.

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