Molecular Interactions between Ribosomal Proteins. Evidence for Specificity of Interaction between Isolated Proteins†

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ABSTRACT: The proteins S2, S3, S5, and S10 from the 30S ribosomal subunit of *Escherichia coli* were studied by analytical ultracentrifugation to characterize them in solution and to determine whether isolated protein–protein interactions exist. Such interactions, if specific, may therefore bear some relationship to the spatial organization of the subunit structure. It was found that protein S2 self-associates to a slight extent and that solution mixtures of S2 and S3 contain only enough dimeric species to account for the S2 dimer. Hence, no observable interaction was detected between S2 and S3. Solution mixtures of the proteins S5 and S10 revealed a species of molecular weight greater than either protein. The proposal is that S5 and S10 interact with an association equilibrium constant of $7.6 \times 10^5\ M^{-1}$ at 3° in a Tris buffer at pH 7.4. It was also shown that a solution with a 1:1:1 mixture, by mass, of S3, S5, and S10 contained a species possessing a molecular weight consistent with a simple ternary complex of the three proteins.

It has been shown previously that the two proteins, S3 and S5, isolated from the 30S ribosomal subunit of *Escherichia coli*, do not self-associate in buffers which promote active ribosomal functions. When a mixture of S3 and S5 is analyzed in the analytical ultracentrifuge, a molecular complex is formed with a free energy of association of −7.25 kcal/mol (Rohde et al., 1975). The lack of self-association of either protein would indicate that this complex is specific for the combination of S3 and S5, but the question might be raised as to whether either protein can interact with any protein in the ribosome to form other paired complexes or even higher order entities. This study presents data to further demonstrate that the S3–S5 complex is possibly unique to the structure of the subunit. Evidence is presented that indicates the proteins do not complex randomly with other ribosomal proteins, and that at least one specific ternary complex may be formed.

Materials and Methods

All of the materials and procedures utilized in this study were discussed by Rohde et al. (1975).

Results and Discussion

This study is concerned with the properties of ribosomal proteins S2, S3, S5, and S10. To investigate any association phenomena of mixtures of these proteins by sedimentation equilibrium analysis, it is first necessary to determine the molecular weight of the individual proteins and the degree of self-association, if any. Molecular weights were determined in the analytical ultracentrifuge by the method of sedimentation equilibrium in two solvents: 6 *M* Gdn·HCl[1] was used to obtain the molecular weights under conditions where self-association mediated by noncovalent interactions would be minimized; and TMK buffer was used to confirm the values obtained in Gdn·HCl and to detect self-association properties.

Table I presents the molecular weights of proteins S2 and S10 determined by sedimentation equilibrium and by sodium dodecyl sulfate electrophoresis using a modified method of Weber and Osborn (1969) previously reported (Rohde et al., 1975). The data for proteins S3 and S5 (Rohde et al., 1975) are also included. The numbers reported from the 6 *M* Gdn·HCl experiments are uncorrected for preferential solvation and are expected to be slightly higher than those from TMK experiments. The difficulties in obtaining reliable molecular weights by dodecyl sulfate electrophoresis have been discussed previously (Rohde et al., 1975), thus, the most reliable estimates are taken to be those values reported in TMK buffer. Nevertheless, there is excellent agreement between the methods even without these considerations.

The identification of proteins S2 and S10 was established in the same manner as discussed previously (Rohde et al., 1975). The protein S2 exhibited only a single band upon gel electrophoresis in the presence of urea or dodecyl sulfate. The fraction containing S10 under the same conditions exhibited a band that migrated where S10 should be and a second band which migrated where the protein S5 migrates. From the isolation procedures it is expected that a contaminant of the fraction containing S10 would be S5. The composition ratio, S10/S5, was estimated to be approximately 3:1 from an observation of the intensity of Amido Black stain in the two bands. Since the sample was small, further steps to remove S5 from S10 were not performed.

The amino acid composition of the protein S2 studied here correlated well with that reported by Craven et al. (1969). The correlation coefficient calculated in the manner discussed previously (Rohde et al., 1975) was found to be 0.991. The amino acid composition of S5–S10 mixture studied here provided a correlation coefficient of 0.958 when compared with the amino acid composition of S10 reported by Craven et al. (1969). The correlation coefficient

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[1] Abbreviations used are: Gdn·HCl, guanidine hydrochloride; TMK, standard buffer containing 0.03 *M* Tris, 0.02 *M* $MgCl_2$, and 0.35 *M* KCl (pH 7.4).

Table I: Molecular Weight Data.[a]

Protein	Sedimentation Equilibrium		Dodecyl Sulfate Electrophoresis
	TMK	Gdn·HCl	
S2	25,100 ± 1000 (2)[b]	26,900 ± 500 (2)	26,200 ± 1000 (5)
S3	21,600 ± 1400 (9)	22,600 ± 500 (5)	25,100 ± 900 (7)
S5	17,500 ± 1600 (8)	18,000 ± 2100 (13)	19,100 ± 700 (5)
S10	*d*	13,600 ± 700 (3)[c]	13,500 ± 1200 (5)

[a] Number of determinations in parentheses. [b] Undergoes self-association or covalent dimer present. [c] Result of extrapolation because sample also contains S5. [d] Higher order association occurring in presence of S5.

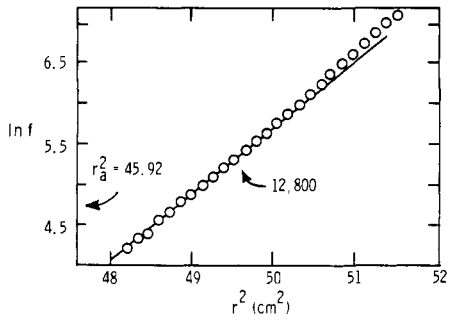

FIGURE 1: Logarithm of the fringe displacement (in microns) vs. the square of the radial position. Sample contained a mixture of S5 and S10 in 6 *M* Gdn·HCl. Conditions were: 44,000 rpm; 25°; column height, 4 mm; total initial protein concentration, 0.25 mg/ml. The indicated molecular weight was obtained from the slope of the line in the figure.

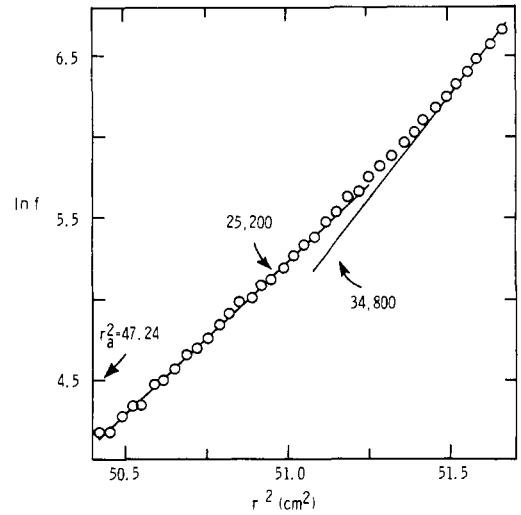

FIGURE 2: Logarithm of the fringe displacement (in microns) vs. the square of the radial position. Sample contained S2 in TMK buffer containing 0.1 *M* 2-mercaptoethanol. Conditions were: 36,000 rpm; 10.9°; column height, 3.3 mm; initial protein concentration was 0.078 mg/ml. The indicated molecular weights were obtained from the slope of the lines in the figure.

for the mixture compared to a synthetic mixture of S5–S10 in a mass ratio of 1:3 was computed to be 0.974. A correlation coefficient is considered to be reasonable when it is 0.97 or greater.

There were two difficulties associated with determination of the molecular weights of S2 and S10 utilizing the method of sedimentation equilibrium. (1) Since the fraction containing S10 also contained S5, the solution was heterogeneous and an unambiquous molecular weight was not directly obtainable from the data. It was observed that in the solvent 6 *M* Gdn·HCl, the limiting slope (Figure 1) in the lower concentration region of a plot of the logarithm of the observed fringe displacement vs. the square of the radial position could be utilized to report the number in the second column of Table I for protein S10. A similar procedure was not utilized to extract the molecular weight for S10 from the data of the S5–S10 mixture in TMK for it became evident that a third species was present in that solvent. The molecular weight of S10 was taken to be 13,000 (less than the value reported in 6 *M* Gdn·HCl due to assumed preferential interactions unaccounted for in the determination in that solvent (Lee and Timasheff, 1974) in TMK buffer). (2) Although S2 was determined to be pure, self-association was indicated in TMK buffer. Protein S2 in this buffer shows evidence of a higher molecular weight species when sedimentation equilibrium data are plotted as the logarithm of observed fringe displacement vs. the square of the radial position (Figure 2). Hence, an extrapolation procedure was used again to ascertain the molecular weight of S2 in TMK. This increase in molecular weight might be due to either noncovalent self-association of the type seen between the subunits of many oligomeric enzymes or may be of a covalent nature through the formation of intermolecular disulfide bonds involving the cysteine residues seen to be present in S2 as has been reported elsewhere (Craven et al., 1969). Since the protein S2 does contain cysteine in its amino acid sequence, disulfide induced dimerization could be the source of the higher molecular weight species. The buffer contained 0.1 *M* 2-mercaptoethanol and normally would be expected to interfere with the formation of intermolecular disulfide bonds. It is conceivable, however, that the environment of the cysteine residues on the surface of the protein is conducive for the stabilization of the oxidized state of a specific cysteine residue. The noncovalent interactions that may provide the driving force to stabilize the second species are eliminated in 6 *M* Gdn·HCl–0.1 *M* 2-mercaptoethanol and a single species with a molecular weight of 26,900 (Table I) is observed.

The molecular weights in Table I are in general agreement with the variations reported by Dzionara et al. (1970).

Analysis of Heterogeneous Samples in TMK. Sedimentation equilibrium data, from experiments in which more than one species was suspected to be present, were analyzed using the direct search fitting procedure developed in this laboratory (M. F. Rohde and K. C. Aune, in preparation). This method calculates the best fit (minimization of average absolute value of the residuals) between the experimental data and any proposed simple model of two to four species, and provides reasonable estimates of the meniscus concentration of each of the species present. The meniscus concentrations can be used directly to calculate association equilibrium constants for interacting mixtures (Rohde et al., 1975). Appropriate integration over the whole cell pro-

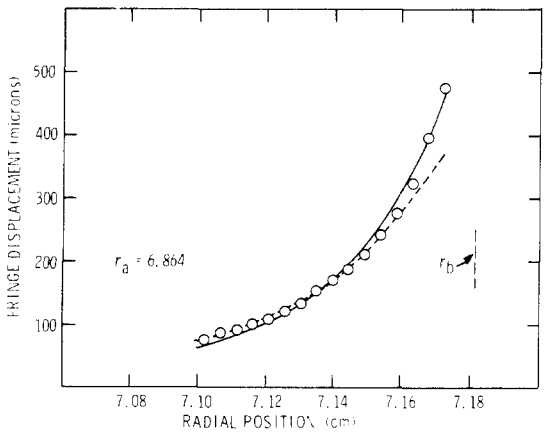

FIGURE 3: The fringe displacement (in microns) vs. radial position. Sample contained a mixture (1:3 by mass) of S5 and S10 in TMK buffer. Conditions were: 39,460 rpm; 3°; column height, 3.2 mm; total initial protein concentration was 0.074 mg/ml. The dashed line is the fit obtained assuming only two species present in the system. The solid line is the fit obtained assuming that the complex, S5–S10, is also present.

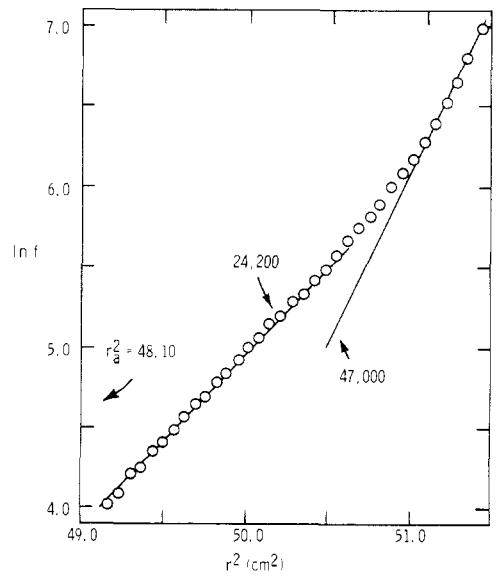

FIGURE 4: Logarithm of the fringe displacement (in microns) vs. the square of the radial position. Sample contained a mixture of S3, S5, and S10 in TMK buffer. Conditions were: 28,000 rpm; 3°; column height, 2.5 mm; initial concentration of each protein was 0.073 mg/ml. The indicated molecular weights were obtained from the slope of the lines in the figure.

vides the total amount of each species present in the cell at equilibrium. The percentage of mass as component j in the system is given by

$$\%M_j = \frac{100\int_a^b f_j \mathrm{d}r^2}{\int_a^b f \mathrm{d}r^2} \quad (1)$$

where

$$f = \sum_{i=1}^{N} f_i \quad (2)$$

and f_i is the fringe displacement of component i in the system containing N species bounded by the meniscus at a radial position $r = a$ and the base of the cell at a radial position $r = b$. For mixtures of protein, this latter calculation provides a check on the reliability of the model and the fit, and for self-associating systems it may be used to determine the degree of self-association.

It was indicated above that samples of S2 in TMK showed evidence of the formation of a species with molecular weight greater than that of monomeric protein and that two sources of this high molecular weight species were considered: a dimer could be formed from either a noncovalent, mass action, concentration-dependent self-association, or from covalent intermolecular disulfide bonds. Two independent sedimentation equilibrium experiments with S2 in TMK buffer at speeds of 35,600 and 36,000 rpm generated two data sets. These data sets were analyzed with the direct search fitting program assuming two species present: monomeric S2 with molecular weight 25,000 and dimeric S2 with molecular weight 50,000. The average deviations between the calculated curve and the experimental points were 3.9 and 7.7 μ, which are well within the expected error due to plate reading inaccuracies. A calculation of the mass percentage of the monomer and dimer for each run gave the result that in both, 13% of the protein mass was present as the dimer. A calculation of an apparent association equilibrium constant, based on a noncovalent interaction model, gave values of 6.6×10^3 and 5.7×10^3 M^{-1}.

Variation of the loading concentration and applied centrifugal force can provide a means of testing the two models of dimer formation, but usually the test is ambiguous when the second species represents only 13% of the mass. Since the apparent equilibrium constant of association was small, experiments with heterogeneous systems were performed.

It has been shown that S3 interacts with S5 in TMK (Rohde et al., 1975). It was desirable to test the hypothesis of whether S3 interacts with other ribosomal proteins. When solutions of S2–S3 mixtures in TMK were analyzed by sedimentation equilibrium analysis, it was found that a species of molecular weight greater than either S2 or S3 was present. The molecular weights of S2 and S3 (25,000 and 21,600, respectively) are sufficiently close such that the direct search fitting procedure could not distinguish between a system containing a species of 50,000 or 46,600. A dimer of S3 has already been ruled out from the earlier study (Rohde et al., 1975).

The average residual for four experimental curves was 10.7 μ. Although this number is greater than the error found for the S2 data in TMK, it is still on the same order of magnitude as the plate reading error. The calculations revealed that the amount of dimer present in the S2–S3 system was low, accounting for approximately 4–11% of the mass in the cell, irrespective of the model. Since the value does not differ greatly from what was found in samples of S2 alone, it is reasonable to conclude that any interaction between the proteins S2 and S3 is weak or nonexistent. It was suggested that S2 and S3 might be physical neighbors in the 30S subunit (Kurland, 1972). The results of this study would suggest that if the proteins are physical neighbors, the relationship is due not to significant interactions between S2 and S3, but rather, due to other factors which cause the proteins to be neighbors. It has also been reported (Sun et al., 1974) that S2 and S3 can be chemically cross-linked in the intact ribosomes. The ability to find a cross-linked pair, however, does not necessarily demand that the two proteins be in physical contact. In fact, it should be noted here that the reverse is also true; the chemical modification method employed in their study would be incapable of detecting interactions when the reactive groups on the proteins are in an unfavorable juxtaposition.

When a solution containing a mixture of S10 and S5 in TMK was analyzed, a third species was observed to be present, for the weight average molecular weight near the

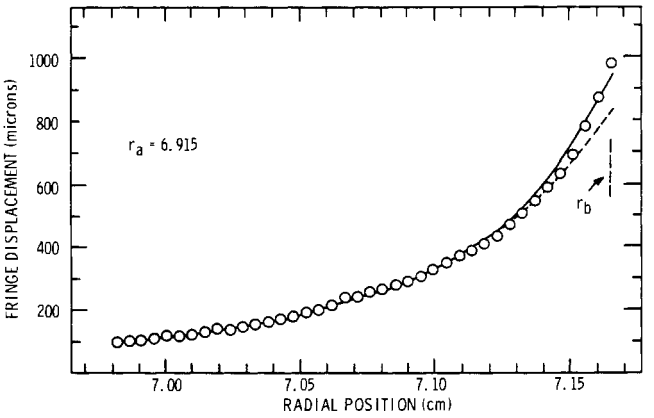

FIGURE 5: The fringe displacement (in microns) vs. radial position. Sample contained a mixture of S3, S5, and S10 in TMK buffer. Conditions were: 25,980 rpm; 3°; column height, 2.5 mm; initial concentration of each protein was 0.073 mg/ml. The dashed line is the fit obtained where a trimeric complex was excluded and complexes of S3–S5 and S5–S10 were present with association equilibrium constants of 5.7×10^5 and 7.6×10^5 M^{-1}, respectively. The solid line is the fit obtained when a trimeric species is present.

bottom of the cell was in excess of 25,000. The two simplest models would be that either S5 is interacting with S10 or S10 is self-associating. The protein S5 has been shown not to self-associate in TMK (Rohde et al., 1975).

The protein S10 does not contain any cysteine residues. Hence, covalent dimers could be ruled out. The fitting procedure could not distinguish between the two models although the variation of the weight average molecular weight supported the former. A fit including a dimeric species of S5–S10 in the system resulted in average residuals of 11.2 and 16.6 μ for two experiments. If the dimeric species is not included, the best fit was with average residuals of 19.5 and 19.2 μ for the same data with deviations from 50 to 100 μ near the bottom of the cell. These results are not consistent with an adequate fit. Therefore, a dimeric species is in the system. The fit is illustrated in Figure 3 for one of the data sets where the solid line is theoretical for three species and the dashed line is theoretical for two species.

If the correct model is one where an S5–S10 dimer is present, an equilibrium constant is calculated for that interaction to be 7.6×10^5 M^{-1} ($\Delta G° = -7.43 \pm 0.28$), at 3° in TMK.

Since the pairs S5–S10 and S3–S5 are involved in interactions, it might be predicted that a ternary complex could form. Sedimentation equilibrium experiments were performed with 1:1:1 mixtures, by mass, of S3, S5, and S10 in TMK buffer at 3°. Figure 4 illustrates the results in terms of a plot of the logarithm of the fringe displacement vs. the square of the radial distance. It is seen, not unexpectedly, that the slope varies throughout the cell. The most important aspect, however, is that the slope near the base of the cell suggests a species is present in the system with a molecular weight greater than any of the dimers. Similar results were obtained in each of three such experiments. The simplest model for a system containing a high molecular weight species is one where a 1:1:1 complex of S3, S5, and S10 is present. Such a system could contain seven species including the three possible types of dimers.

The data from a system containing seven species cannot be uniquely resolved. The imposition of the constraints on the calculation that the two equilibrium constants $K_{3,5}$ and $K_{5,10}$ have the values previously determined removes two

Table II: Parameters Describing S3–S5–S10 System.

Expt. No.	% (S3–S5–S10)[a]	$K_{3,5,10}$[a] (M^{-2})	$\Delta G°_{3,5,10}$[a] (kcal)	Av Residual (μ)
41.25980[b]	24	6.7×10^{11}	−14.9	10.9
41.33450[b]	19	3.0×10^{11}	−14.5	5.4
1045.28000[c]	28	5.2×10^{11}	−14.8	18.8

[a] Computed from the fitting parameters with four degrees of freedom. These numbers are considered to be representative of the system, but not necessarily unique. [b] Same solution analyzed in U.C. equipped with mechanical speed control. [c] Solution analyzed in U.C. equipped with electronic speed control.

unknowns. The solution is still not unique. No information is presently available about the possibility of an S3–S10 interaction. The number of degrees of freedom are so great that the presence or absence of an S3–S10 dimer could not be established by curve fitting. Therefore, the fit achieved in Figure 5 by the direct search procedure is essentially identical for the presence [assumes $\ln K_{3,10} = \frac{1}{2}(\ln K_{3,5} + \ln K_{5,10})$] or absence of the S3–S10 dimer and has an average residual of 10.9 μ. In either case, approximately 24% of the mass present in the system is the ternary complex. This can be considered significant and provides strong evidence for at least the formation of a ternary complex. The dashed line in Figure 5 is theoretical and is a best fit obtained (the average residual = 17.7 μ) without ternary complex formation. A satisfactory fit could not be achieved, particularly near the base of the cell. The results of the three data sets are reported in Table II. Also reported in the table is the value of the equilibrium constant and Gibbs free energy for the formation of ternary complex. It should be emphasized that these latter numbers fit the data, but are not necessarily unique. It should be noted, however, that the unitary free energy for trimer formation is −17.2 kcal compared to −19.3 kcal, the sum of the unitary free energies for S3–S5 and S5–S10 formation. This might suggest that the trimer formation results only from the two dimeric interactions and not from a closed trimeric structure. A cyclic complex, involving an S3–S10 interaction as well, could be expected to provide a much lower free energy for complex formation.

The fact that a ternary complex forms when S3, S5, and S10 are mixed gives credence to that model where S5 and S10 interact, although it does not prove the interaction, for a test of the presence or absence of a S3–S10 dimer has not been made. Since S2 does not interact measurably with S3, the negative result may be considered as further evidence that specific interactions are involved in these studies rather than indiscriminate aggregation of proteins. The results reported here are consistent with the order of assembly of the proteins in the 30S ribosomal subunit as discussed elsewhere (Held et al., 1974). This study demonstrates, however, that if one desires a complete description of the mechanism for the formation of the ribosomal subunits, one must consider the isolated interactions between the components that comprise the system. This is in addition to other forms of indirect information such as is obtained in reassembly and cross-linking studies.

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The Uncatalyzed Rates of Enolization of Dihydroxyacetone Phosphate and of Glyceraldehyde 3-Phosphate in Neutral Aqueous Solution. The Quantitative Assessment of the Effectiveness of an Enzyme Catalyst†

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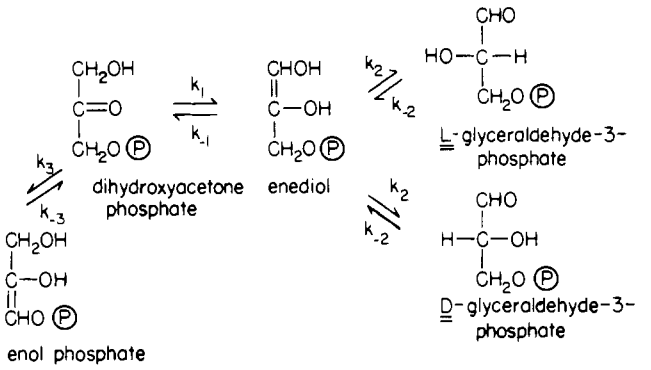
ABSTRACT: By a combination of methods involving enzyme-catalyzed reactions and classical iodination techniques it has been possible to obtain all the relevant rate constants for the uncatalyzed interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate via their common enediol intermediate. These rate constants are compared with those for the individual steps of the triosephosphate isomerase catalyzed reaction, and a quantitative picture of the effectiveness of the enzyme as a catalyst has been delineated. It is apparent that the enzyme increases the enolization rate of dihydroxyacetone phosphate by a factor of more than 10^9 over that of the uncatalyzed reaction.

The kinetics of the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate catalyzed by the glycolytic enzyme triosephosphate isomerase (EC 5.3.1.1.) are well understood, and the rate constants for all the kinetically significant steps of the catalyzed reaction have been determined (W. J. Albery and J. R. Knowles, unpublished work). From these data, it is evident that the isomerase has developed to the point where further evolutionary refinement would have no effect, since the interconversion is diffusion controlled.

In order to compare the catalyzed rates of this simple one-substrate–one-product reaction with the rates of the uncatalyzed process, we need to know the rates of enolization of the two reactants, and the equilibrium percentage of their common enediol. The two reports in the literature of these rates (Reynolds et al., 1971; Grazi et al., 1973) are conflicting, and it seems not to have been appreciated that the enol from glyceraldehyde 3-phosphate is the same as one of the two possible enols from dihydroxyacetone phosphate (Reynolds et al., 1971). Since there is good evidence that the enzyme-catalyzed reaction also involves this common enediol as an intermediate (Rieder and Rose, 1959), it is of some interest to find how the free energy profile (which is simply a convenient way of illustrating and summarizing the rate and equilibrium constants) for the uncatalyzed reaction compares with that for the maximally efficient interconversion catalyzed by triosephosphate isomerase. We report here the results obtained for the rates of the enolization reactions, determined both by the classical method involving iodination of the enediol, and also by a novel series of enzymic conversions that confirm and extend the iodination data.

Scheme I. Possible Enolization Reactions Involving Dihydroxyacetone Phosphate and Glyceraldehyde 3-Phosphate.



The species involved in the enolization of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate are outlined in Scheme I, which shows the five distinct species and the six rate constants that connect them. Dihydroxyacetone phosphate may enolize between C-1 and C-2 to give the common enediol, and between C-3 and C-2 to give the enol phosphate. Both D- and L-glyceraldehyde 3-phosphate may enolize to the common enediol. Not shown in Scheme I are the hydrated forms of each of the triosephosphates, but even though these forms are significant in aqueous solution (41% for dihydroxyacetone phosphate and 96.2% for glyceraldehyde 3-phosphate: Gray and Barker, 1970; Reynolds et al., 1971), the rate constants for hydration–dehydration are approximately two orders of magnitude larger than those for enolization, and do not therefore affect the results obtained.

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