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Enzymatic and fluorescence studies of four single-tryptophan mutants of rat testis fructose 6-phosphate,2-kinase:fructose 2,6-bisphosphatase

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Abstract

In order to determine environments around four tryptophan residues, located in the N-terminus, in the kinase and in the phosphatase domains of rat testis Fru 6-P,2-kinase:Fru 2,6-bisphosphatase, mutant enzymes containing a single tryptophan were constructed by site-directed mutagenesis. The kinetic constants of these mutant enzymes were similar to those of the wild-type enzyme. The sum of the fluorescence intensities of the enzymes was 1.5× that of the wild-type enzyme, and Trp 299, Trp 64, Trp 15, and Trp 320 contributed 38%, 28%, 17%, and 17%, respectively. The fluorescence polarization of the wild-type enzyme was significantly lower than any of the mutant enzymes, suggesting proximity of two tryptophan residues in the wild-type enzyme. The polarization in the presence of Fru 6-P affected only Trp 15, which suggested that it is located near the Fru 6-P binding site, but Trp 64 is not. Inactivation of both enzyme activities and unfolding of these enzymes in guanidine were monitored by activity assays and fluorescence intensities and maxima. Both Fru 6-P,2-kinase and Fru 2,6-bisphosphatase activities of all these enzymes were inactivated between 0.7 and 1 M guanidine. Enzymes containing Trp 64 or Trp 15 showed biphasic fractional unfolding curves, but those of Trp 299 or Trp 320 showed gradual steady changes. Fluorescence quenching by iodide indicated that Trp 64 was not accessible and that other Trp residues were only slightly accessible to solvent. These results suggest that all the Trp residues are in heterogeneous environments and that none are exposed on the protein surface.

Keywords: bifunctional enzymes; Fru 6-P,2-kinase:Fru 2,6-Pase; glycolysis; intrinsic fluorescence; tryptophan in protein

A bifunctional enzyme, Fru 6-P,2-kinase:Fru 2,6-bisphosphatase (Fru 2,6-Pase), catalyzes the synthesis (Fru 6-P + ATP ⇌ Fru 2, 6-P_2 + ADP) and the degradation (Fru 2,6-P_2 → Fru 6-P + P_i) of Fru 2,6-P_2, the most potent activator of phosphofructokinase. Different mammalian tissues contain tissue-specific isozymes of the bifunctional enzyme and these isozymes have differing relative activities of kinase and phosphatase. These isozymes are homodimers comprised of subunits with M_r ranging from 54,000 to 60,000. The amino acid sequences of the liver (Darville et al., 1987; Algaier & Uyeda, 1988; Lively et al., 1988), skeletal muscle (Crepin et al., 1989), heart (Sakata & Uyeda, 1990), and testis (Sakata et al., 1991) enzymes have been determined. The amino acid sequences of both catalytic domains are well-conserved among these isozymes, but the amino and carboxyl termini are completely different. These differences in the terminal peptides appear to contribute to the different relative activities. The activity ratio of the kinase and phosphatase can be regulated also by phosphorylation/dephosphorylation of either N- or C-terminus. Phosphorylation of the N-terminus of the liver enzyme results in inhibition of the kinase and activation of the phosphatase (van Schaftingen et al., 1981; El-Maghrabi et al., 1982; Furuya et al., 1982). On the other hand, phosphorylation of the C-terminus of the heart enzyme results in activation of the kinase without affecting the phosphatase activity (Kitamura & Uyeda, 1987; Kitamura et al., 1988). Such regulation of opposing enzyme activities in a reciprocal manner involves close communication between catalytic domains and is manifested via inter- and intrasubunit interactions. Understanding of the regulatory mechanism requires elucidation of these interactions.

Denaturation and renaturation studies can provide useful information concerning subunit interactions. Previously (Tominaga et al., 1994) we investigated reversible unfolding of testis Fru 6-P,2-kinase:Fru 2,6-Pase in guanidine using intrinsic tryp-

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tophan fluorescence and the enzyme activities as probes. We presented evidence that the unfolding reaction may follow a four-state transition from dimer → partially unfolded dimer → monomer → unfolded monomer. The rat testis enzyme contains four tryptophan residues (Sakata et al., 1991), Trp 15 at the N-terminus, Trp 64 in the kinase domain, and Trp 299 and Trp 320 in the phosphatase domain. In order to gain more insight into the environment around these Trp residues, which are distributed in the catalytic domains as well as at the N-terminus, and also to gain more detailed information regarding the unfolding process, we have constructed mutated forms of the enzymes containing either no Trp or a single Trp at each location, the other Trp residues having been converted to Phe. The kinetic parameters and conformational dynamics of these variants were investigated using the intrinsic fluorescence as a spectroscopic probe, and the results are presented in this report.

Results

Kinetic parameters of Trp mutant enzymes

As shown in Table 1, the kinetic constants of the mutant Fru 6-P,2-kinase were not altered significantly. The K_m^{ATP} and V_{max} values of the kinases were nearly the same as those of the wild-type enzyme. The K_m^{F6P}, however, increased from 35 μM to 45–80 μM, or maximally twofold.

The K_m^{F26P2} and the V_{max} of the Fru 2,6-Pase in these derivatives increased at most about twofold. These results suggested that none of the Trp in the enzyme was essential for substrate binding or for catalytic activity and that the replacement of three out of four Trp residues or all the Trp residues with Phe did not cause significant conformational changes in the enzyme's active site.

Fluorescence spectra and relative yields

Emission spectra of the wild-type Fru 6-P,2-kinase:Fru 2,6-Pase and the Trp mutants (1.82 μM subunits), when excited at 295 nm are shown in Figure 1. The fluorescence intensity of W299 was the highest followed by W64, W15, and W320. The fluorescence maxima of W299 and W64 were 334 nm, whereas those of W15 and W320 were 336 nm and 337 nm, respectively. Figure 1 also shows that the sum of fluorescence of these mutant enzymes was more (1.8×) than that of the wild-type enzyme. These spectra, however, were uncorrected for instrument response functions, which include a significant polarization bias (for example, Wood's anomaly, which results in the shoulder near 380 nm) of the SLM holographic grating monochrometers (Jameson, 1984).

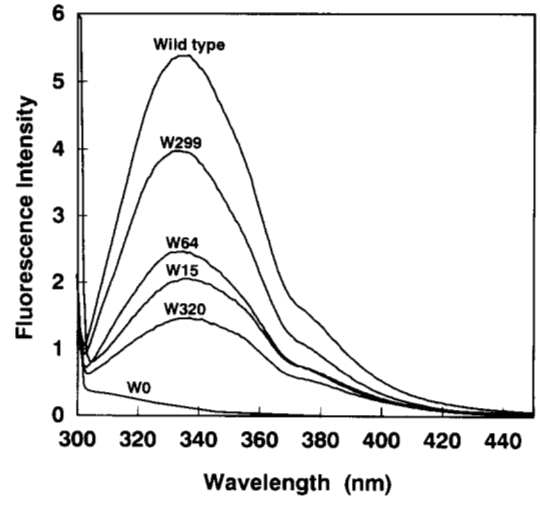

Fig. 1. Intrinsic tryptophan emission spectra of rat testis Fru 6-P,2-kinase:Fru 2,6-Pase and mutant enzymes. All solutions contained 0.9 μM enzymes in 50 mM Tris/phosphate, pH 7.5, 0.5 mM EDTA, and 2 mM dithiothreitol. Fluorescence spectra were determined as described in the Materials and methods. Excitation wavelength was 295 nm. Spectra were uncorrected for detector response function. Spectra are, from the top, wild type (WT), W299, W64, W15, W320, and W0.

To estimate the relative yields of each tryptophan residue more precisely, the total emission at wavelengths >300 nm was observed through WG305 cut-on filters, upon excitation at 280 nm. In these measurements, a SLM-8000C spectrofluorometer equipped with calcite prism polarizers was utilized. The exciting light was polarized parallel to the vertical laboratory axis, and the emission was observed through a polarizer oriented first parallel ($I_\parallel$) and then perpendicular ($I_\perp$) to the excitation polarizer. The total emission intensity (I_T) was then calculated from $I_T = I_\parallel + 2I_\perp$; these data were obtained using an L-format in which the polarization bias of the filter/PMT combination was negligible. The results are summarized in Table 2. The sum of the intensities of the mutant enzymes was 1.5× that of the wild-

Table 1. *Kinetic constants of the mutant Fru 6-P,2-kinase and Fru 2,6-Pase*

	Fru 6-P,2-kinase			Fru 2,6-Pase	
	K_m^{ATP} (μM)	K_m^{F6P} (μM)	V_{max} (milliunits/mg)	K_m^{F26P} (nM)	V_{max} (milliunits/mg)
Wild type	70 ± 26	35 ± 11	80 ± 12	46 ± 13	17 ± 1
W-0	60 ± 25	80 ± 23	80 ± 14	60 ± 15	30 ± 2
W64	80 ± 21	70 ± 19	80 ± 10	108 ± 20	38 ± 2
W15	60 ± 16	50 ± 10	75 ± 8	41 ± 14	22 ± 2
W320	50 ± 21	45 ± 25	75 ± 20	72 ± 10	36 ± 2
W299	70 ± 23	70 ± 27	75 ± 23	54 ± 11	36 ± 2

Table 2. *Fluorescence intensity of the wild-type and Trp mutant enzymes*[a]

	Fluorescence intensity (arbitrary units/mg)	Contribution (%)
Wild type	28.9 ± 0.2	100
W15	7.1 ± 0.3	17
W64	12.0 ± 0.2	28
W299	16.5 ± 0.2	38
W320	7.3 ± 0.3	19
	42.9 ± 1.0	100

[a] Total fluorescence emission (>300 nm) was determined at excitation wavelength of 280 nm. Standard errors calculated based on three determinations.

type enzyme, Trp 299 and Trp 64 contributed 38% and 28%, respectively, comprising 60% of the total emission. Trp 15 and Trp 320 contributed 17% each.

Fluorescence polarization

The steady-state polarizations (upon 300 nm excitation) for each mutant and the wild-type enzyme in the presence and the absence of Fru 6-P are given in Table 3. The polarization of the wild-type enzyme was significantly lower than any of the mutant enzymes both in the presence and in the absence of Fru 6-P. The effect of Fru 6-P was seen only with the W15 enzyme.

Unfolding and inactivation by guanidine

The emission spectra upon 295 nm excitation was used to follow the unfolding of the enzymes in the presence of varying concentrations of guanidine. The fluorescence intensities of W15 and W64 at 334 nm decreased linearly to 0.5 M–0.7 M, whereas those of the wild type, W299, and W320 remained constant over the same range of low guanidine concentration (Fig. 2A). However, the intensity of the latter enzymes dropped sharply between 0.5 M and 0.8 M guanidine, and more gradually above these guanidine concentrations. These results may indicate that the regions of Trp 64 and Trp 15 in the kinase domain began to unfold as soon as the enzyme was exposed to dilute guanidine, but those regions in the phosphatase domain were stable until guanidine concentration exceeded 0.5 M. The previous results with the DANS-labeled wild-type enzyme indicated that the fluorescence polarization changes drastically between 0.7 M and 2 M quanidine in protein concentration-dependent manner, indicating dissociation of dimer to monomer (Tominaga et al., 1994).

The sum of fluorescence intensities of the mutant enzymes was much larger than that of the wild-type enzyme below 2 M guanidine, but became identical above 2 M (Fig. 2A, inset).

The fluorescence maximum of all the enzymes shifted from 334–357 nm to 352 nm with increasing guanidine concentration (Fig. 2B), indicating that the tryptophan(s) were being exposed to a new environment. Sharp transitions in the fluorescence maxima of W64, W299, and the wild type were observed in 0.4–0.8 M guanidine.

Inactivation of Fru 6-P,2-kinase activity of the wild-type and the mutant enzymes in varying concentrations of guanidine is shown in Figure 3A, B, C, D, and E. The kinase activities of all these enzymes were inactivated by the same ranges of guanidine concentrations with a half maximum inactivation at 0.8 M, except for W299, which had a half maximum inactivation at approximately 0.7 M. Slight activation of the kinase activity of W15 occurred at below 0.8 M guanidine. The reason for this activation is unclear.

In contrast to the current results, previous data (Tominaga et al., 1994) showed that Fru 6-P,2-kinase was inactivated below 0.5 M guanidine. This difference was attributable to the enzymes being stored frozen for more than a few weeks. Apparently, the duration of storage and repeated freezing-thawing made the kinase activity more susceptible to denaturation. Thus, in all the current studies, freshly prepared enzymes were used to avoid this situation.

Fru 2,6-Pase of W320 and the wild-type enzymes were more resistant to guanidine inactivation than the other enzymes (Fig. 3); half maximum inactivation of the former enzymes occurred at 1 M guanidine compared to 0.7–0.8 M for the latter enzymes.

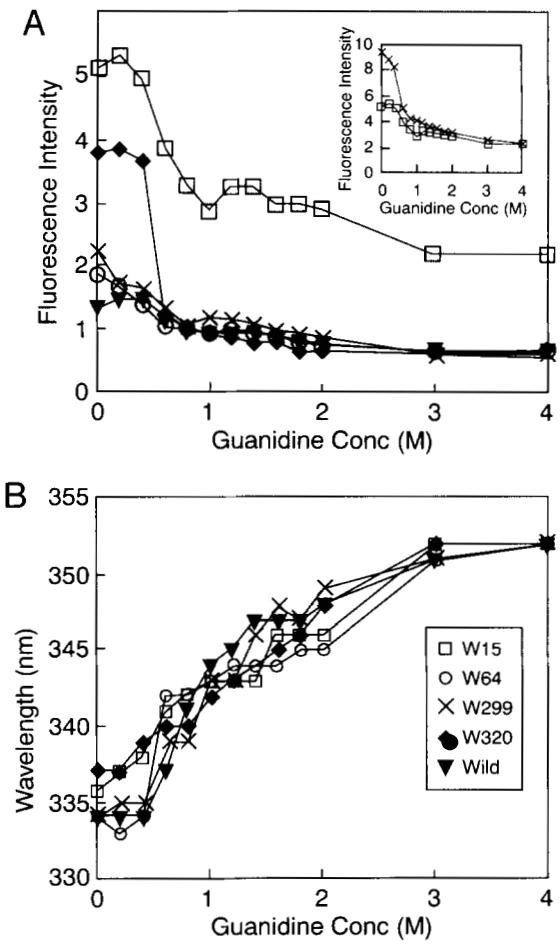

Fig. 2. A: Changes in fluorescence intensity of Fru 6-P,2-kinase:Fru 2,6-Pase in guanidine. Fluorescence intensity of the enzyme (10 μg) in 100 μL was determined at the excitation wavelength at 295 nm and the emission wavelength at 334 nm for WT, W64, and W299, or at 336 nm for W15, and 337 nm for W320. The intensity value of W0 enzyme was subtracted from that of each enzyme. **B:** Shift in emission maximum. Wild type (▼); W299 (X); W64 (○); W15 (□); and W320 (◆). The inset is a comparison of fluorescence intensity of wild type and a sum of four mutants. Wild type (■) and W15+W64+W299+W320 (**X**).

Table 3. *Fluorescence polarization in the presence and absence of Fru 6-P*[a]

	Polarization[b]	
Enzyme	Minus Fru 6-P	Plus Fru 6-P
Wild type	0.260	0.259
W15	0.290	0.310
W64	0.325	0.322
W299	0.302	0.302
W320	0.311	0.310

[a] Excitation wavelength was 300 nm.
[b] Standard deviations are ±0.002.

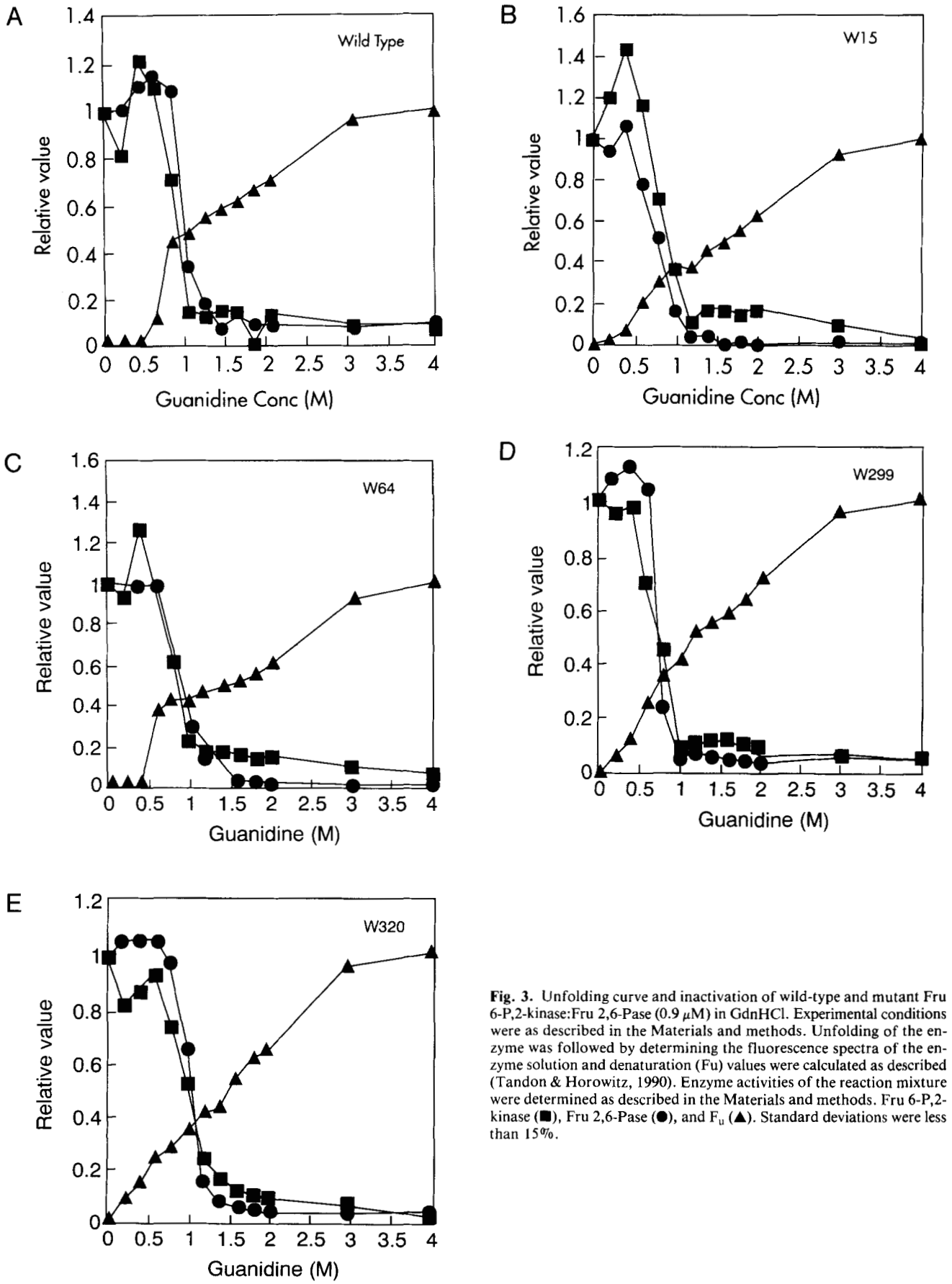

Fig. 3. Unfolding curve and inactivation of wild-type and mutant Fru 6-P,2-kinase:Fru 2,6-Pase (0.9 μM) in GdnHCl. Experimental conditions were as described in the Materials and methods. Unfolding of the enzyme was followed by determining the fluorescence spectra of the enzyme solution and denaturation (Fu) values were calculated as described (Tandon & Horowitz, 1990). Enzyme activities of the reaction mixture were determined as described in the Materials and methods. Fru 6-P,2-kinase (■), Fru 2,6-Pase (●), and F_u (▲). Standard deviations were less than 15%.

Slight activation of the phosphatase of the wild-type, W299, W320, and W0 enzymes were also seen below 1 M guanidine. Among those enzymes, both kinase and phosphatase of Trp 299 were least stable in guanidine.

A fractional unfolding, Fu, was calculated using the equation $Fu = 1 - (R_o - Rd)/(Rn - Rd)$, where R_o is the observed fluorescence intensity,[3] and Rd and Rn are the intensities in the presence and absence of 4 M guanidine, respectively. The plots of Fu and the enzyme activities in varying concentrations of guanidine are shown in Figure 3A, B, C, D, and E. The plot of Fu versus guanidine concentration of the wild-type enzyme showed a sharp increase at half-maximal concentration of approximately 0.7 M, which is similar to the previous results (Tominaga et al., 1994). A half maximal inactivation of the kinase and the phosphatase occurred at 0.8 M, close to the first transition in Fu (Fig. 3A). The plot for Trp 15 showed an initial slow rise in the Fu to 0.5 M, a sudden increase up to 1 M and slow and linear increase up to 4 M. The initial unfolding in 0.5 M guanidine was associated with activation of Fru 6-P,2-kinase, and the second phase of the increase in unfolding corresponded to inactivation of both enzyme activities (Fig. 3B). The Fu plot of W64 showed a curve similar to the wild-type enzyme, except half-maximum concentration of the first transition occurred at 0.5 M (Fig. 3C). Activation of the kinase was observed up to 0.5 M guanidine, but, above this concentration, inactivation occurred with a rapid increase in the Fu. Unlike those of W15 or W64, the Fu curves of both W299 and W320 (Fig. 3D,E) rose continuously without any sharp transition.

Iodide quenching

Iodide, a large polar anion thought to have access only to surface-exposed Trp residues, can be useful in determining a tryptophan residue's extent of solvent accessibility (Lehrer, 1971; Eftink & Ghiron, 1976). Iodide quenching of the wild-type and the mutant enzymes was examined, and the results were analyzed using the Stern-Volmer equation, $F_o/F = 1 + K_{SV}[Q]$, where F_o and F are the fluorescence intensities in the absence and the presence, respectively, of quencher, Q, and K_{SV} is the Stern-Volmer constant (Lehrer & Leavis, 1978). In using this equation, we assumed that there was no static quenching. As shown in Figure 4A, the wild-type enzyme and W64 showed essentially no quenching by iodide, suggesting that all the Trp residues in these enzymes were completely buried and not accessible to the solvent. However, the plot of the denatured W64 in the presence of 4 M guanidine had a positive slope, indicating that this Trp became accessible to the quencher. Among the other mutant enzymes, W299, W320, and Trp 15 showed increasing slopes of the SV plots. These results were further analyzed using the modified Stern-Volmer equation, $F_o/\Delta F = 1/(f_a \cdot K_{SV}[Q]) + 1/f_a$, where F_o is the fluorescence in the absence of quencher; ΔF is the difference in the fluorescence in the absence and the presence of a quencher; f_a is the fraction of accessible tryptophan,

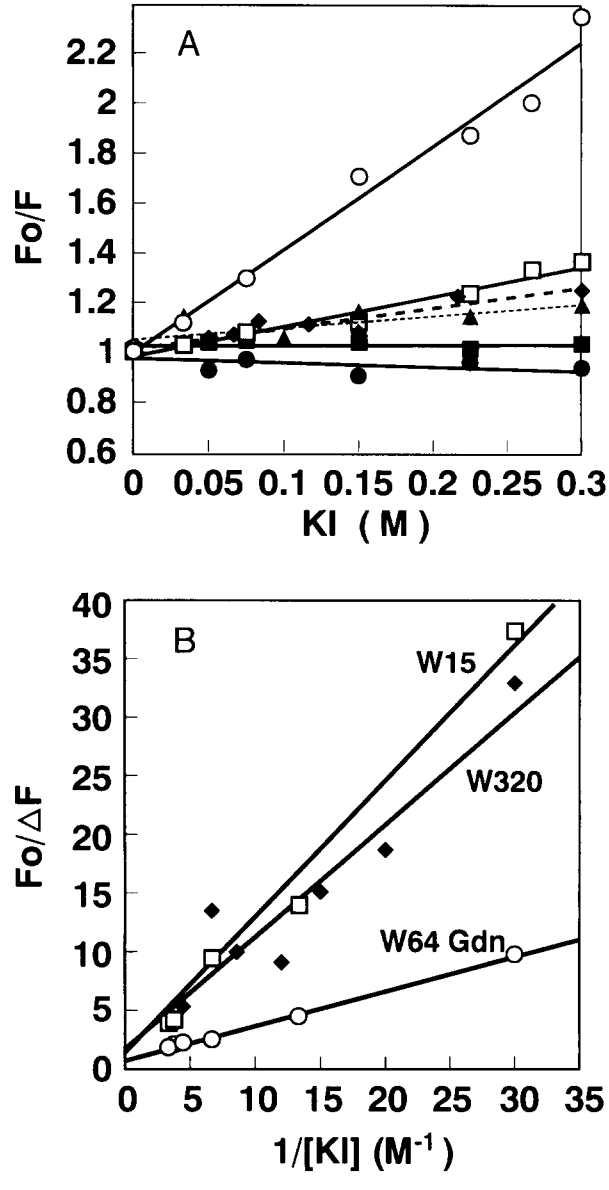

Fig. 4. Stern-Volmer plot of iodine quenching of wild-type and mutant Fru 6-P,2-kinase:Fru 2,6-Pase. Fluorescence intensity of the enzyme (0.9 μM) was determined in the reaction mixture containing increasing concentrations of KI as described in the Materials and methods. The excitation and fluorescence maxima were 295 nm and 334 nm for the wild type (■), W64 (●), and W299 (▲), and 336 nm for W15 (□), and 337 nm for W320 (◆), W64 in 4 M guanidine (○). **A:** Stern-Volmer plot. **B:** Modified Stern-Volmer plot.

and K_{SV} and $[Q]$ are the same as above (Lehrer, 1971). The results in Figure 4B show that the f_a values of W15, W320, and W64 in guanidine were approximately 1, implying that these Trp residues were accessible to quencher. However, the same plots of Trp 299 and the wild-type enzyme were nonlinear (data not shown) and the corresponding f_a values were 0.17 and 0, implying that these Trp residues were less accessible. K_{SV} values for W15, W320, W299, the wild type, and W64 calculated from the plots were 1.1 M^{-1}, 1.0 M^{-1}, 0.63 M^{-1}, 0.02 M^{-1}, and 0.0, re-

[3] We should note that Fu plot presumes a two-state unfolding model. We realize the unfolding-refolding of the enzyme involves more than two states of the enzyme conformation (Tominaga et al., 1994). However, Fu equation was used for comparison in normalizing the denaturation curves of these mutants. Moreover, the changes in the fluorescence intensity only detect two states, including dissociation to monomers and unfolding of the monomer (Tominaga, 1994).

Table 4. *cDNAs of the wild-type rat testis Fru 6-P,2-kinase:Fru 2,6-Pase primers used for oligonucleotide-directed mutagenesis*

Strain	DNA sequence[a]						
	K	K	I	W^{15}	M	P	Y
W15 Wild type	5′-AAG	AAG	ATC	TGG	ATG	CCA	TAC-3′
				**			
F15 Mutant	5′-AAG	AAG	ATC	TTT	ATG	CCA	TAC-3′
				F			
(W15F) Oligonucleotide	3′-TTC	TTC	TAC	AAA	TAC	GGT	ATG-5′
	Y	L	N	W^{64}	I	G	V
W64 Wild type	5′-TAC	CTC	AAC	TGG	ATT	GGC	GTG-3′
				**			
F64 Mutant	5′-TAC	CTC	AAC	TTT	ATT	GGC	GTG-3′
				F			
(W64F) Oligonucleotide	3′-ATG	GAG	TTG	AAA	TAA	CCG	CAC-5′
	L	L	N	W^{299}	I	G	V
W299 Wild type	5′-CTG	AAG	GTC	TGG	ACG	AGC	CAG-3′
				**			
F299 Mutant	5′-CTG	AAG	GTC	TTT	ACG	ACC	CAG-3′
				F			
(W299F) Oligonucleotide	3′-GAC	TTC	CAG	AAA	TGC	TGC	GTC-5′
	Y	E	Q	W^{320}	K	V	L
W320 Wild type	5′-TAT	GAG	CAG	TGG	AAG	GTC	CTC-3′
				**			
F320 Mutant	5′-TAT	GAG	CAG	TTT	AAG	GTC	CTC-3′
				F			
(W320F) Oligonucleotide	3′-ATA	CTC	GTC	AAA	TTC	CAG	GAG-5′

[a] The DNA sequence of wild-type rat testis Fru 6-P,2-kinase:Fru 2,6-Pase was changed at the positions marked by ** to yield the desired mutant cDNAs. Amino acid residues substituted are given in parentheses. Numbers superscripted on the amino acid sequence are the residue numbers of wild-type enzyme.

spectively, compared to 4.1 M^{-1} for the completely unfolded Trp 64 (Table 4).

Discussion

The results presented in this paper show that replacement of three or all four Trp residues of rat testis Fru 6-P,2-kinase:Fru 2,6-Pase with Phe does not cause large perturbations of the protein's conformation and functions. This conclusion was based on the following observations: (1) there was no alteration in the elution pattern from the ion exchange chromatography or the affinity chromatography; (2) there were no large changes in the kinetic constants of both kinase and phosphatase except for approximately twofold increase in the K_m for Fru 6-P of the kinase and K_m for Fru 2,6-P_2 and V_{max} of the phosphatase in some of the enzymes; and (3) the denaturation curves of the mutant enzymes and the wild-type enzyme in guanidine were similar. Therefore, it is reasonable to assume that the overall conformation of the wild-type enzyme and the mutant enzymes containing three of four Trp residues changed to Phe were similar.

These observed changes in the kinetic parameters and the other properties were comparable or smaller than those seen in the other mutant enzymes (Tominaga et al., 1993; Abe & Uyeda, 1994). For example, introduction of the consensus amino acid sequence (Arg Arg Ala Ser) for protein kinase A in the N-terminus region, a regulatory domain of the bifunctional enzyme, causes the similar or lower changes in the K_m^{F6P} of the kinase (Abe & Uyeda, 1994). Deletion of 24 or 30 amino acids of the N-terminus causes a much larger increase in K_m^{F6P} and a large drop in k_{cat} of the kinase (Tominaga et al., 1993). Nevertheless, some small differences in surface exposed Trp residues were observed as discussed later.

The differences in the fluorescence spectra among the mutants (Fig. 1) per se do not permit us to estimate the degree of solvent exposure for the tryptophan residues. Although a general tendency toward "bluer" emissions from buried tryptophans has been noted in the literature, sufficient exceptions exist (Longworth, 1983) to warrant caution in drawing firm conclusions about the degree of solvent exposure and polarity of a tryptophan residue's environment simply on the basis of emission maxima. The microscopic details of the environment such as the extent of polarizability, the presence of water molecules, and the specific interactions between the indole ring and polar groups in the protein will all influence the maximum emission wavelength.

The sum of the quantum yields of the individual Trp was 1.5× that of the wild-type enzyme, suggesting that the yield of specific tryptophan residues in the mutants was different than in the wild type. This difference could arise from conformational alterations or may be due to Trp–Trp energy transfer in the wild type, which does not occur in the mutant. The polarization results also bear on this issue. Specifically, the polarization of each individual tryptophan residue in the mutant enzyme was relatively high, ranging from 0.290 to 0.325. The limiting or intrinsic polarization of tryptophan upon excitation at 300 nm is 0.405 (Valeur & Weber, 1977). Considering the molecular mass of the

protein (110 kDa), the previously determined rotational relaxation time of DANS-labeled enzyme (180 ns) (Tominaga et al., 1994), and the relatively short lifetimes of the tryptophan residues (<7 ns in all cases; data not shown), we have concluded that each residue experienced some local mobility (in the absence of local motion and given the considerations listed above one can calculate, using the Perrin equation, that polarizations would be in the range of 0.37 or higher). More detailed time-resolved anisotropy studies are being conducted currently to quantify more precisely the extent of motion demonstrated by each tryptophan residue in the mutant protein. A striking observation, however, was the distinctly lower polarization (0.260) noted for the wild-type enzyme compared to any mutant protein. This difference suggested the following possibilities: (1) one or more of the tryptophan residues in the wild-type protein exhibit higher local mobility than as the respective mutants; (2) tryptophan-tryptophan energy transfer occurs in the wild type; (3) exciton coupling between Trp residues. Given the observations concerning the sum of the relative yields of the mutant enzymes compared to the wild type, we favor the Trp-Trp energy transfer hypothesis. We note that the failure of self-energy transfer, which occurs upon red-edge excitation of tryptophan, becomes pronounced at excitation wavelengths >305 nm (Weber, 1960). We also note that 280 nm excitation, which was utilized to facilitate the collection of the entire emission, also excites tyrosine residues. To avoid tyrosine excitation, one must excite at 300 nm or higher, which results in a significantly reduced absorption by tryptophan, and hence weak signal, and also a Raman band near 337 nm. Using 300 nm excitation, one would also have to use a cut-on filter that blocked the exciting light and hence the first part of the emission spectra. We note firstly, however, that, upon excitation at 280 nm, there are no evident shoulders in the spectra near 305 nm, indicative of tyrosine emisson, and secondly, because the number of tyrosine residues in the wild-type and mutant proteins are constant, any small contribution of tyrosine to the emission should be more or less the same in all cases. Hence, 280 nm excitation would introduce a significant bias in the relative quantum yield calculation only if a significantly different extent of tyrosine to tryptophan energy transfer occurs in the different proteins. Fluorescence lifetime measurements that will address this issue more completely are being conducted presently.

The previous results (Tominaga et al., 1994) of the iodide quenching of the wild-type enzyme yielded a nonlinear Stern-Volmer plot, suggesting heterogeneous environment around the Trp residues. The use of the single Trp-containing enzymes provided additional information regarding environment surrounding each Trp residue. The present results confirmed the previous observation that only Trp 15 and Trp 320 are readily accessible to iodide, suggesting that Trp 64 and Trp 299 are more buried in the native enzyme.[4] Trp 15, which is in the N-terminus, was the most exposed among all the Trp residues, which is not unexpected. However, the Stern-Volmer constant of even Trp 15 was relatively small, suggesting that it is not freely exposed to solvent and that it is probably binding to the enzyme surface. This conclusion is supported by the previous observation (Tominaga et al., 1994) that the N-terminus peptide is important in the subunit-subunit interaction. Trp 299 and Trp 320 were also somewhat accessible to iodide, suggesting that this region of the Fru 2,6-Pase domain was accessible to the solvent. Interestingly, however, Trp 64 was not accessible at all to iodide and appeared to be completely buried. In the primary sequence of the enzyme, Trp 64 is located near the ATP binding region.

The observation that Trp 299 and Trp 320 in these mutant enzymes were quenched by iodide to a small extent may indicate an alteration of this conformation, somewhat exposing those Trp residues to solvent. This altered conformation might be caused by the changes in three Trp to three Phe residues. All these mutant enzymes have the loss of Trp 64 in common, and it is possible that Trp 64 is essential for a native compact conformation of the enzyme. The unfolding in guanidine also supported those conclusions. Based on the Fu plots (Fig. 3), significant unfolding of Trp 299, Trp 320, and Trp 15 occurred in dilute guanidine (<0.5 M), even though both enzyme remained active, but no unfolding of Trp 64 and the wild-type enzymes was observed under the same conditions. Sharp transition during unfolding was observed in the latter enzymes only when guanidine was increased from 0.5 to 1 M. The previous fluorescent polarization measurements (Tominaga et al., 1994) demonstrated that the enzyme dissociates in the same range of guanidine, which is closely associated with inactivation of both enzymes (Fig. 3A,C). These results suggested that Trp 64 may be at or near subunit interface. Moreover, these results confirm our previous conclusion that partial unfolding occurs before inactivation. The present results with these mutants suggested that the initial unfolding occurred in the Fru 2,6-Pase domain before the dissociation.

Trp 15 is located in the N-terminal peptide of rat testis Fru 6-P,2-kinase:Fru 2,6-Pase. It is interesting that Fru 6-P increased the polarization of only Trp 15, suggesting that this Trp is localized near the Fru 6-P binding domain of the Fru 6-P,2-kinase active site. This idea is further supported by other circumstantial evidence. Deletion of the N-terminal peptide of the enzyme increases K_m for Fru 6-P, and the deleted enzyme is more susceptible to dissociation and inactivation (Tominaga et al., 1993). Fru 6-P protects the enzyme against heat and denaturant inactivation and also enhances the rate of renaturation of guanidine or urea-denatured enzymes (Tominaga et al., 1994). The fact that the polarization of Trp 64 was not affected by Fru 6-P suggests that this Trp residue is not located near the Fru 6-P binding site.

Materials and methods

Homogeneous recombinant rat testis Fru 6-P,2-kinase:Fru 2,6-Pase was prepared from *Escherichia coli* BL21 (DE3) carrying the RT2K/pT7-7 plasmid as described previously (Tominaga et al., 1993). Muscle phosphofructokinase was prepared as described (Uyeda et al., 1978). Restriction enzymes and bacteriophage T4 DNA ligase were purchased from Pharmacia LKB Biotechnology Inc. (Piscataway, New Jersey). MutaGene M13 in vitro mutagenesis kit was purchased from BioRad Laboratories (Hercules, California). Guanidine-HCl (GdnHCl) was purchased from Sigma Chemical Co (St. Louis, Missouri). Electrophoretic grade acrylamide was obtained from BioRad (Richmond, California). All the solutions used for fluorescence studies were passed through a 0.22-μm filter. All other chemicals were reagent grade and obtained from commercial sources.

[4] We should note, however, that the local charge environment, i.e., proximity of negative or positive charges, can affect local concentration of iodide ion and hence affect the extent of quenching.

Preparation of mutants by site-directed mutagenesis

Plasmid RT2K/pT7-7, containing the rat testis Fru 6-P,2-kinase:Fru 2,6-Pase (RT2K) cDNA cloned in a pT7-7 vector (Sakata et al., 1991), was digested with *Xba* I and *Hin*d III, and an isolated 1.7-kb fragment was ligated into the *Xba*-*Hin*d III site of M13 mp18 (RT2K/M13). The ligation mixture was used to transform *E. coli* JM109 cells selecting Amp^r. The phage harboring RT2K/M13 was prepared from selected transformants and transfected into *E. coli* CJ236 (dut^-, ung^-). The uracil-containing single-stranded DNA was purified from CJ236 cells and used as a template for in vitro mutagenesis. Oligonucleotide-directed mutagenesis was performed as described by Kunkel (1985) using the Muta-Gene M13 in vitro mutagenesis kit (BioRad). Single Trp to Phe (W → F) mutant RT2K cDNAs were constructed using a synthetic oligonucleotide encoding partial sequence of each mutant (Table V). The phosphorylated oligonucleotide primer (25 ng) and the uracil-containing single-stranded recombinant DNA (200 ng) were incubated at 70 °C for 5 min in 10 μL of 20 mM Tris-HCl, pH 7.4, containing 2 mM $MgCl_2$ and 50 mM NaCl. The mixture was cooled to 30 °C gradually (1 °C/min) and left on ice for 5 min. The annealed mutagenic primer was extended in the presence of 5 mM $MgCl_2$, 0.5 mM dNTP (dATP, dGTP, dTTP, and dCTP), 2 mM dithiothreitol, and 1 mM ATP with 1 unit of T7 DNA polymerase, and then the mutated strand was ligated with 3 units of T4 DNA ligase during incubation for 5 min at 25 °C followed for 30 min at 37 °C. The resultant heteroduplexed DNA was used to transform competent *E. coli* MV1190. The mutant derivatives were identified by DNA sequencing. Double mutant RT2K cDNAs (W15F/W64F/RT2K/M13mp18, and W299F/W320F/RT2K/M13mp18) were constructed with the same method. A triple Trp mutant was constructed from a combination of one double and one single Trp mutant DNA. For example, a mutant termed W320, containing a single Trp at #320 with all three Trp residues changed to Phe, was constructed by digesting the W15F/W64F/RT2K/M13mp18 and W299F/RT2K/ pT7-7 DNAs with *Nde* I and *Bam*H I (Fig. 5A). The fragment containing F15 and F64 and that containing F299W, W320/pT7-7 were ligated. The Trp-less mutant, in which all Trp residues were changed to Phe, was prepared from *Bam*H I and *Hin*d III-digested DNA fragments containing F299-F320 and F15-F64/pT7-7 as shown in Figure 5B.

GdnHCl treatment

Fru 6-P,2-kinase:Fru 2,6-Pase (0.1 mg/mL) in 50 mM Tris-phosphate, pH 7.5, 0.5 mM EDTA, 2 mM dithiothreitol (Buffer A),

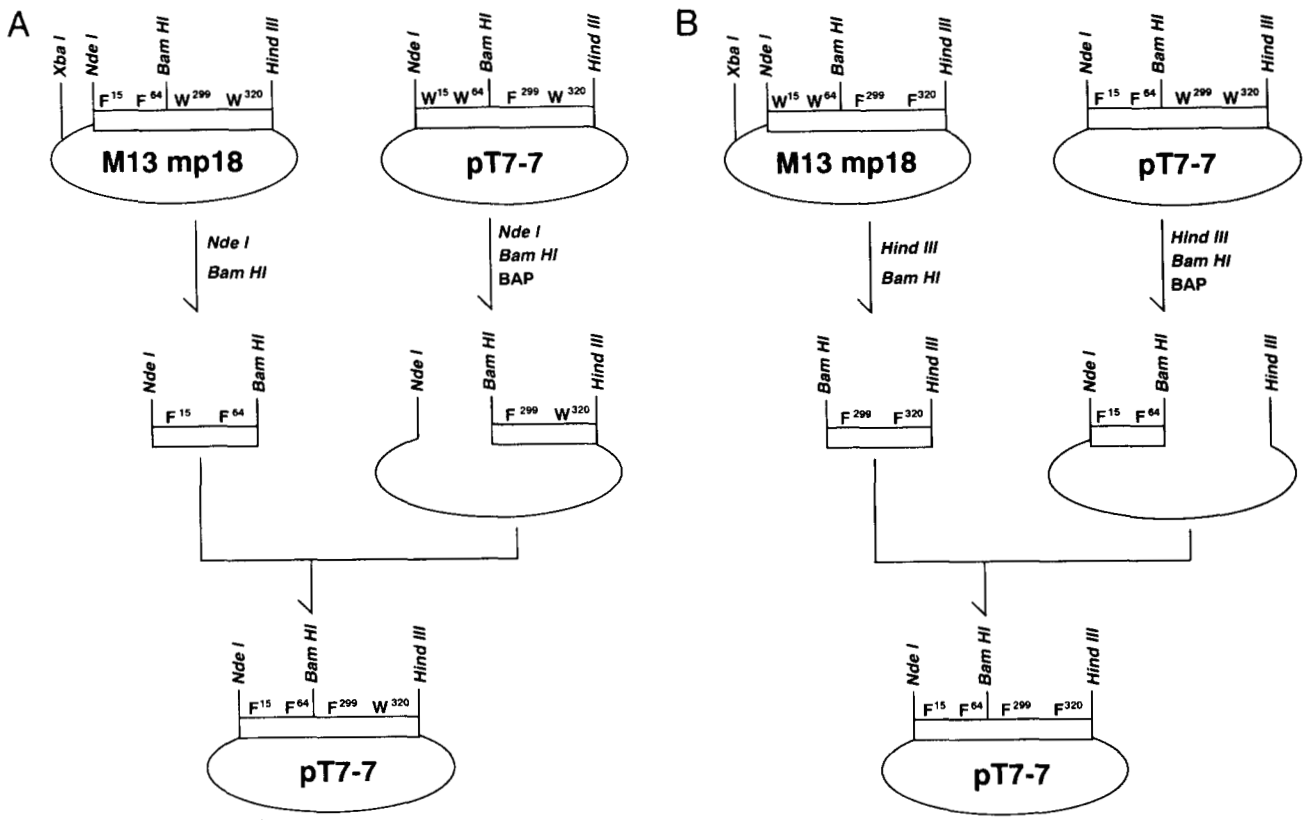

Fig. 5. Schematic description of the construction of recombinant vectors for expression of rat testis Fru 6-P,2-kinase:Fru 2,6-Pase mutants. **A:** Construction of W320/pT7-7. Replicative form of M13mp18 harboring RT2K double mutant (W15F/W64F) was digested with *Nde* I and *Bam*H I. The *Nde* I/*Bam*H I fragment containing W15F/W64F mutation was ligated to *Nde* I/*Bam*H I site of W299F/pT7-7 vector treated with bacterial alkaline phosphatase (BAP). **B:** Construction of W0 mutant. A *Bam*H I/*Hin*d III fragment (1.2 kb) of W299F/W320F/RT2K cDNA was cloned to *Bam*H I/*Hin*d III site of W15F/W64F/RT2K/pT7-7 expression vector.

and varying concentrations of GdnHCl was incubated at 0 °C for 1 h. Aliquots were removed for enzyme activity assays and also for fluorescence measurements. The stock solution of GdnHCl solution (5 M) was neutralized to pH 7.5 with KOH.

Fluorescence spectra

Fluorescence spectra were obtained at 25 °C using an Aminco-Bowman Series 2 spectrofluorometer (SLM-Aminco/Champaign, Illinois). Excitation and emission bandpasses were 4 nm. For intrinsic tryptophan fluorescence, 295 nm excitation was utilized. Emission spectra were collected from 300–450 nm. Spectra were corrected for buffer background, but not for instrument response functions.

Fluorescence quenching studies

The reaction mixture contained, in a final volume of 0.1 mL, 50 mM Tris/phosphate, pH 7.5, 0.5 mM EDTA, 2 mM dithiothreitol (Buffer A), varying concentrations of potassium iodide, the selected concentration of GdnHCl, and the enzyme at 0.1 mg/mL (0.9 μM). Additionally, appropriate amounts of KCl were added to maintain constant ionic strength. The emission spectra of the mixtures were then obtained. To prevent I_3^- formation, 0.05 mM sodium thiosulfate was included in the stock potassium iodide solution (2 M), and this mixture was prepared fresh each time. The reaction mixture was incubated at 25 °C for 5 min.

Fluorescence intensity

Fluorescence intensity was measured using an SLM-8000C spectro-fluorometer (SLM-Aminco/Champaign, Illinois). The fluorescence intensity of the intrinsic protein emission was determined as follows. The enzyme (0.2 mg/mL, 1.8 μM) was excited at 280 nm, and emission at wavelengths >300 nm was viewed through a Schott KV320 cut-on filter; the excitation bandpass width was 4 nm.

Fluorescence polarization

Fluorescence polarization measurements were performed on an ISS K2 spectrofluorometer (ISS, Inc./Champaign, Illinois), using a Spectra-Physics model 2045 argon-ion laser as the excitation source. The sample was illuminated at 300 nm and emission at wavelengths >340 nm (to avoid the water Raman peak) was viewed through a Schott WG 345 cut-on filter. Polarization measurements were conducted at 25 °C.

Assay method for Fru 6-P,2-kinase

The reaction mixture contained in a final volume of 0.1 mL, 100 mM Tris/HCl, pH 7.5, 0.1 mM EDTA, 5 mM ATP, 1 mM Fru 6-P, and 10 mM $MgCl_2$. The reaction was started with the addition of Fru 6-P,2-kinase and incubated at 30 °C for 3, 6, and 9 min. Aliquots (10 μL) were transferred to 90 μL of 0.1 N NaOH, and the diluted solution was heated at 80 °C for 1 min to stop the reaction. Appropriate aliquots of the heated reaction mixture were then assayed for Fru 2,6-P_2 as described (Uyeda et al., 1981). One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of Fru 2,6-P_2/min under these conditions.

Assay method for Fru 2,6-Pase

The fluorometric coupled assay was described previously (Tominaga et al., 1994). The reaction mixture contained in a final volume of 0.6 mL, 100 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 50 μM NADP, 17 μM Fru 2,6-P_2, 0.4 unit of desalted glucose 6-P dehydrogenase, and 1 unit of phosphoglucose isomerase. The reaction was initiated with the addition of Fru 2,6-Pase, and was followed at room temperature fluorometrically at excitation and emission wavelengths of 354 nm and 452 nm, respectively. One unit of activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of Fru 6-P (as NADPH production)/min under these conditions.

Protein concentration assay

Protein concentration was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard. We found previously (Sakakibara et al., 1985) that the extinction coefficient of the colored product based on the dry weight of the pure enzyme was identical to that of bovine serum albumin.

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