Additional Kinetic Distinctions Between Normal Pyruvate Kinase and a Mutant Isozyme From Human Erythrocytes. Correction of the Kinetic Anomaly by Fructose-1,6-diphosphate

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Several cases have been reported in which chronic hemolytic anemia was thought to be secondary to the same mutant isozyme of erythrocyte PK. In crude hemolysates from one of these cases, defective kinetic characteristics of the isozyme were found to be completely correctable in vitro by low concentrations of the PK activator, fructose-1,6-diphosphate. The patterns of response to the activator further distinguished this isozyme from normal PK and suggested that the isozymes in these cases, previously thought to be identical, more probably represent three subtly distinct mutant forms.

Inherited deficiency of erythrocyte pyruvate kinase (PK), EC 2.7.1.40, is often definable in chronic hemolytic anemias of the nonspherocytic type. In such cases, quantitative reductions of cellular PK are usually indicted etiologically, but qualitative abnormalities of the enzyme molecule also exist which may be associated with a clinical syndrome otherwise indistinguishable from classical, homozygous PK deficiency. In four such instances, a PK isozyme with anomalous kinetics, pH optimum and functional stability was demonstrable, which we arbitrarily designated PK2 in distinction to the wild enzyme referred to as PK1.1 This mutant isozyme had an apparent Michaelis-Menten constant (Km) for one of its substrates, phosphoenolpyruvate (PEP), 10-fold greater than normal values, and thus was presumed to function poorly at PEP concentrations as low as those normally existent within mature human erythrocytes.

One of the patients so afflicted was reinvestigated to evaluate the effect of a known PK activator, fructose-1,6-diphosphate (FDP),2 on the kinetic characteristics of the aberrant isozyme. Studies reported herein demonstrated that low concentrations of FDP in vitro were capable of correcting the kinetic ab-
normality completely and that the patterns of response to this activator provided a further mode of distinction among PK isozymes.

MATERIALS AND METHODS

Details of the patient's clinical history have been presented in a previous report in which he was designated Propositus 4, Kindred D. He was 19-years old, of English extraction, and had a lifelong history of marginally compensated hemolytic anemia characterized by hyperbilirubinemia, reticulocytosis, and splenomegaly. Erythrocyte autohemolysis was elevated, conforming to the Selwyn-Dacie Type II pattern with partial correction by glucose and complete correction by adenosine triphosphate (ATP). Erythrocyte 2,3-diphosphoglycerate concentrations were twice normal mean values. Glycolytic enzyme activities were all normal or elevated commensurate with the degree of reticulocytosis, but red cell PK was found to have a $K_m$ (PEP) of 1.31 mM, compared to a normal mean of 0.13 mM. The $K_m$ for adenosine diphosphate (ADP) was normal.

Venous blood from the patient and a normal control was anticoagulated with heparin, refrigerated with ice and air expressed from Hanover, N.H., to Los Angeles. The specimens were processed and assayed within 36-48 hours. Erythrocytes were prepared as free of leukocytes as possible by sedimentation in polyvinylpyrrolidone and filtration through nylon wool prior to multiple saline washes. The enzymatic assay of Bucher and Pfleiderer, as modified in an earlier report, was used to measure PK activities in freshly prepared crude hemolysates. The assay system contained 8 mM MgSO$_4$, 75 mM KCl, 0.2 mM reduced nicotine adenine dinucleotide (NADH), 6 units per ml. crystalline lactate dehydrogenase, and either 0.4 or 2.0 mM ADP in 8.3 mM triethanolamine hydrochloride buffer at a final pH of 7.2 ± 0.1. PEP concentrations for $K_m$ determinations were varied between 0.05 and 16.7 mM. Enzyme units (U) were defined as the number of micromoles of NADH oxidized each minute by $10^{10}$ erythrocytes at 37°C and were determined by monitoring absorbance changes at 340 nm.

RESULTS

PK activity in crude hemolysates was found to vary as a function of PEP concentration in the manner shown (Fig. 1). Maximum reaction velocities ($V_{max}$) for normal PK$_1$ average 2.7 U in our laboratory, standard deviation (SD) = 0.5 U, and are usually achieved with 1.0 to 2.0 mM PEP. By contrast, activity of mutant PK$_2$, though markedly diminished at lower substrate levels, eventually reached a higher maximum (4.2 U), but only after PEP concentration was elevated to 5.0 mM.
Substrate concentrations coinciding with half maximal activities directly defined $K_m$ (PEP) for erythrocyte PK: 0.16 mM for PK1 in the shipped control specimen, and 1.96 mM for proband PK2. With control hemolysates, addition of 0.1 mM FDP to the reaction medium elevated $V_{max}$ little if at all (< 10%), but activities at suboptimal PEP concentrations were significantly enhanced, thereby decreasing $K_m$ (PEP) to about half its original magnitude. This pattern of response to FDP activation has been a remarkably consistent finding in our laboratory. Twenty-nine subjects with normal PK kinetics have yielded a mean $K_m$ (PEP) of 0.18 mM (SD = 0.04 mM) which decreased to a mean of 0.07 mM (SD = 0.01 mM) under the influence of FDP. The direction of this change was invariable in each instance, thereby enhancing the statistical significance.

With PK2 in proband lysates, FDP shifted the curve dramatically from a skewed sigmoid to a normal rectangular hyperbola. $V_{max}$ increased slightly (< 20%), and $K_m$ (PEP) decreased tenfold from 1.96 to 0.21 mM, a value within our upper range of normal.

Enhanced ADP availability did not alter the divergent patterns of response to FDP activation by the two isozymes (Fig. 2). Virtually the same relative changes in $V_{max}$ and $K_m$ (PEP) were observed. PK1 in normal hemolysates achieved optimal reaction rates above 2.0 mM PEP, whereas PK2 did not reach an activity plateau until substrate concentration was approximately 13.0 mM PEP. In the presence of FDP, however, PK2 activity was maximal at much lower substrate concentrations (3 mM PEP), while $V_{max}$ remained relatively unchanged, thereby decreasing $K_m$ (PEP) from 4.1 to 0.6 mM. Under the same conditions, PK1 $K_m$ (PEP) was halved to 0.15 mM PEP. These results support in part the observation of Campos et al.⁸ that $K_m$ (PEP) of erythrocyte PK is a dependent variable of ADP concentration, suggesting that a definitive order of substrate binding is prerequisite to catalysis. There is then
preliminary evidence to support a similar conclusion regarding the mutant PK2 isozyme.

The effect of FDP on $K_m$ (PEP) for either normal PK1 or mutant PK2 was not observed to alter significantly within the concentration range of 0.01 to 1.00 mM FDP. We detected no FDP effect on $K_m$ (ADP) with either isozyme.

**DISCUSSION**

These observations are quite compatible with the molecular model for FDP modulation of PK proposed by Koler and Vanbellinghen. Their data supported the existence of at least two catalytic sites on erythrocyte PK. They hypothesized that attachment of either PEP or FDP to one catalytic (rather than allosteric) site substantially increases substrate affinity at the second catalytic site, and that affinity for PEP is quite low if neither site is occupied. We would infer that in the case of PK2 an even lower primary affinity for PEP exists, caused perhaps by a genetically induced molecular disconformation which may be corrected in large measure by combination with FDP in small amounts or with PEP in substantially higher concentrations. Studies are in progress to determine whether other distinguishing characteristics of PK2, such as pH optimum and electrophoretic mobility, may be similarly modified by FDP.

Our present findings are in sharp contrast to those of Munro and Miller, who restudied another of the propositi included in our original collaborative report (Propositus 1, Kindred G). Using partially purified erythrocyte PK2 from this patient, they found $V_{max}$ and $K_m$ (PEP) both markedly increased following exposure to 1.0 mM FDP. Yet their results with partially purified normal PK1 were essentially the same as our observations with crude normal hemolysates. Thus these two families, originally thought to harbor the same aberrant PK isozyme, may in point of fact be quite different.

Additionally, Boivin et al. have reported two cases of hemolytic anemia also interpretable as PK2 anomalies. When they assayed simple hemolysates, they found $V_{max}$ to be normal and $K_m$ (PEP) elevated but insensitive to alteration by FDP. Partial purification of the enzyme, however, resulted in a preparation which responded well to FDP, the elevated $K_m$ (PEP) reverting to normal values in the presence of the activator.

Extraction procedures are known to alter PK kinetic characteristics from those observed in unrefined lysates. Until all of these cases are evaluated under more comparable conditions, using both crude hemolysates and preparations purified by uniform techniques, it remains uncertain whether they are truly distinct or identical.

It is becoming clearer that hemolytic anemias due to PK deficiency may be quite polymorphous and that additional parameters of activation kinetics must be considered now in attempting to distinguish among mutant PK isozymes. Some consideration must be tendered soon to a system of nomenclature, perhaps similar to that used for glucose-6-phosphate dehydrogenase variants, which will simplify their identification.
ACKNOWLEDGMENT

We are grateful to Dr. O. Ross McIntyre for providing us with additional blood specimens from his patient; to Julie Wittenberg, Anneliese Merlino, Ernesto Guereque, and Klaus Kürschner for technical contributions; and to Ruth Exley for manuscript assistance.

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