Charge and Solvation Effects in Anion Recognition Centers: An Inquiry Exploiting Reactive Arginines†

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ABSTRACT: Following a long-standing suggestion of Riordan et al. [Riordan, J. F., McElvany, K. D., and Borders, C. L., Jr. (1977) *Science 195*, 884−885], we sought to exploit chemically activated arginines as probes to characterize the microenvironmental effects in enzymes that mediate the recognition of anionic substrates. A micellar simulation study establishes that octylguanidine (OGn) becomes chemically activated upon incorporation into both cetyltrimethylammonium bromide (CTAB) and Triton X-100 micelles and that the activations correlate with the pK_a diminutions induced in its guanidinium group by the effects of electrostatic or nonelectrostatic nature as reflected in the results of pH and salt titration experiments. Next, a protein modification study establishes that the modifiable arginines in a number of enzymes also have diminished pK_a's, again due to effects of electrostatic or nonelectrostatic nature as reflected in the results of pH and salt titration experiments. Warwicker's finite difference Poisson−Boltzmann algorithm [Warwicker, J. (1992) *J. Mol. Biol. 223*, 247−257] is applied to several of the enzymes with available crystal structure coordinates, and indeed, their chemically activated arginines are found to be in an electrostatic microenvironment that can diminish their pK_a's, with the magnitudes of these diminutions matching closely the diminutions measured experimentally. Finally, the chemically activated arginines are examined with respect to their atomic atmosphere and are thus found to occur in a local microenvironment that would facilitate their roles as anion anchors. Thus, electrostatic and solvation effects are found to be critical determinants of the arginine role as an anion anchor.

In linking catalytic activity with electronic structure in enzymes, ionizable group pK_a's provide a vitally important handle (*1*, *2*). Being microenvironmentally sensitive, the pK_a's are useful sensors of solvation or electrostatic effects that may be crucial to the catalytic function of an enzyme (*3*, *4*). An ionizable group of strategic importance as an enzyme catalytic element is the guanidinium group (GnH^+) of arginine. Arginine has an unusual propensity to be an active site element (*5*), and an exceptional efficiency as the counterion for carboxyl and phosphoryl anions, donating multiple H bonds to these functional groups and forming geometrically well-defined salt bridges of exceptional stability (*6*−*8*). The intrinsically high pK_a of GnH^+ (*9*), however, is an impediment to the pK_a measurement in the assessment of microenvironmental effects in arginine's role as an enzyme catalytic element. Microenvironmental effects diminishing arginine pK_a, on the other hand, do appear to be operative and are reflected in the chemically activated nature of active site arginines in several important enzymes acting on anionic substrates (*10*−*12*). Indeed, it has been a well-recognized notion in biochemical literature that the microenvironmental effects mediating anion recognition could be the effects causing arginines to become chemically activated (*4*, *11*, *13*). Thus, "activated" arginines could be useful sensors of enzyme catalytic effects, helping to characterize both their structural origins and functional importance.

A recently proposed mechanism rationalizing the vast body of experimental observations on the arginine−PGO[1] reaction (*14*−*16*), providing the basis for its exploitation to probe active site effects, is outlined in Scheme 1. It seems the modification of an arginine begins in a rapid preequilibrium step when GnH^+ adds to the extremely electrophilic and almost completely hydrated aldehydic carbonyl in PGO. The resultant monocarbinolamine **Ia** suffers an intramolecular addition, furnishing the dicarbinolamine **II**. The latter is the primary product in arginine modification and is susceptible

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[1] Abbreviations: PGO, phenylglyoxal; DMAPGO, 4-(dimethylamino)phenylglyoxal; BGn, butylguanidine hydrochloride; OGn, octylguanidine hydrochloride; DDGn, dodecylguanidine hydrochloride; CTAB, cetyltrimethylammonium bromide; SDS, sodium dodecyl sulfate; HK, hexokinase; CK, creatine kinase; PK, pyruvate kinase; 3-PGK(m), muscle 3-phosphoglycerate kinase; MDH(mit), mitochondrial malate dehydrogenase; MDH(cyt), cytosolic malate dehydrogenase; LDH, lactate dehydrogenase; AK1, muscle adenylate kinase; AK3, mitochondrial matrix adenylate kinase; SOD, superoxide dismutase; NAD/NADP, oxidized nicotinamide adenine dinucleotide/phosphate; PEP, phosphenolpyruvate; NAD(H), reduced nicotinamide adenine dinucleotide.

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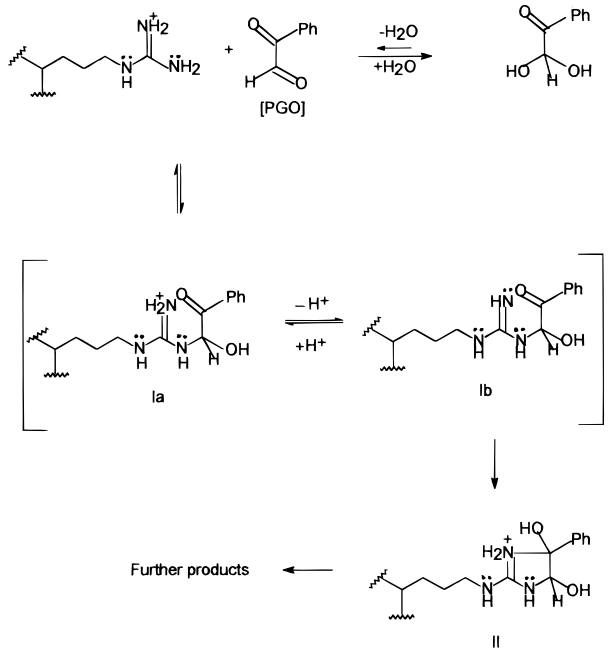
Scheme 1



to alternative fates, such as complexation with buffers (*16*), rearrangement (*15*), or condensation with another molecule of the α-dicarbonyl reagent (*17*), depending on the experimental conditions. The Hammet correlational approach established the sensitivity of the modification rate of an arginine to the substituent electronic effects in PGO, giving a ρ value of close to unity (*16*, *18*). Thus, the nucleophilic attack at aryl ketone, giving the dicarbinolamine **II**, was proposed as the rate-determining step in arginine modification (*18*). Another mechanistically important observation was that the modification is a specific base-catalyzed process, and thus, its rate-determining step, furnishing the dicarbinolamine **II**, involves monocarbinolamine **Ia** in the unprotonated form **Ib**. Thus, the activation of an arginine could be the consequence of effects that diminish the pK_a of monocarbinolamine **Ia**.

The microenvironmental effects diminishing the monocarbinolamine pK_a will be those capable of destabilizing the protonated monocarbinolamine **Ia**, shifting the protropic equilibrium in favor of the unprotonated monocarbinolamine **Ib**. The effects, likely to be a positive electrostatic field and a diminished local polarity or dielectric constant, could be simulated in micellar models and assessed experimentally by using pH and salt titration experiments. Thus, in possibly exploiting activated arginines as kinetic reporters of the microenvironmental effects in anion recognition, we undertake here a four-pronged experimental and theoretical study. First, a micellar simulation study establishes that an arginine side chain model can be activated by the microenvironmental effects that can diminish its pK_a. Next, the activated arginines in a number of enzymes are shown to display diminished pK_a's, due to effects of electrostatic or nonelectrostatic nature as reflected in the results of pH and salt titration experiments. Next, an electrostatic simulation study using the available crystal structure coordinates establishes that the activated arginines in some of the enzymes indeed are in an electrostatic microenvironment that can diminish their pK_a's, the calculated pK_a's being reasonably consistent with the measured pK_a's. Finally, the activated arginines in the protein PDB structures are assessed with respect to their atomic atmosphere and are on this basis found to occur in a local microenvironment that indeed will facilitate their role as anion anchors.

EXPERIMENTAL PROCEDURES

Materials. LDH (porcine H_4 and M_4, rabbit, and chicken muscle), AK1 (porcine and rabbit muscle), MDH (porcine heart mitochondrial and cytoplasmic), PK (chicken muscle), HK (yeast), CK (porcine muscle), 3-PGK (porcine muscle), SOD (bovine erythrocyte), ATP, PEP, ADP, AMP, NAD, NADH, NADP, 3-phosphoglycerate, Hepes, and Bicine were from Sigma Chemicals. [^{14}C]PGO (27 μCi/mmol) was from Amersham. CTAB and Triton X-100 were from Spectrochem (Bombay, India). All other chemicals were of analytical or reagent grade. All solutions and buffers were in triple-distilled deionized water.

Synthesis. The synthesis of PGO and DMAPGO has been described (*18*). BGn, OGn, and DDGn, synthesized as sulfate salts (*19*, *20*), were converted into chlorides by $BaCl_2$ precipitation, purified by ion exchange chromatography, and characterized by elemental and spectroscopic analysis.

Spectrometric, Radioactive, and pH Measurements. All pH measurements were with a combination electrode precalibrated against primary pH standards at pH 4.0, 7.0, and 9.2. Spectrometric measurements were carried out with a Shimadzu UV-265 instrument in a thermostated cuvette compartment at 27 ± 0.1 °C. Radioactive measurements were carried out with a Rack Beta scintillation counter.

Buffers. A number of buffers, notably, borate, bicarbonate, and phosphate, will interact with arginine or react with PGO and manifest activating or deactivating effects in arginine modification (*21*). Hepes and Bicine, being buffers that are inert toward arginine−PGO reaction (*18*), were thus used in this investigation. The requisite pH value in a buffer (50 mM) was initially set with 1 N NaOH or HCl, and the ionic composition was estimated from the pK_a values (*22*). The ionic strength was then set with NaCl to 0.04, 0.09, 0.16, 0.25, 0.49, 0.70, and 1.0, and the minor pH readjustment after the ionic strength was set was made with 1 N NaOH or HCl. The resultant variation in ionic strength (always within ±3%) was allowed. For the pH range of 6.5−8.0, Hepes was the buffer, while for the pH range of 8.5−9.5, Bicine was the buffer.

Guanidine Modifications. PGO and DMAPGO are the modifiers used against alkylguanidines, and PGO is the modifier used against proteins. The choice is dictated by the unfavorable absorbance overlap between PGO and Triton X-100 which interferes in micellar experiments, and between DMAPGO and NAD(H) which interferes in enzyme assays. The kinetic runs for guanidine modification, monitored by the decrease in DMAPGO absorbance at 357 nm and PGO absorbance at 254 nm, were allowed to proceed to completion to correct for the absorbance changes due to the accumulated products. Due to the low intrinsic reactivity of DMAPGO (*18*), most micellar experiments were carried out at pH 9.0, while the variable-pH experiments were carried out with PGO as the modifier.

Micellar Experiments. DDGn was initially assessed as the candidate for micelle incorporation, but it displayed extremely poor water solubility. OGn was assessed next and

displayed adequate solubility as well as the absence of any aggregation or micellarization at ≤40 mM as judged by fluorometric (*23*) and conductometric methods (*24*) validated against Triton X-100 and CTAB.

SDS, CTAB, and Triton X-100 were constituted in Bicine buffer in the presence or absence of OGn (10 mM). An insoluble complex was formed between OGn and SDS, preventing any further studies with this detergent. Thus, further experiments were pursued only with CTAB and Triton X-100. The modification reactions were initiated by the addition of DMAPGO to a pure or mixed detergent solution to a final concentration of 100 μM. When 10 mM solutions of CTAB or Triton X-100 were mixed (a greater than critical micellar concentration for either), a ≈10% decrease in absorbance of DMAPGO occurred almost instantaneously, possibly due to its equilibration into the micellar systems. Hence, an equilibration period of 1 min was allowed after rapidly mixing in DMAPGO before monitoring the kinetic runs. BGn (10−100 mM) and PGO (100 μM) were similarly reacted in Hepes or Bicine buffer, and the kinetic runs were monitored spectrophotometrically. The k_{obs} values were determined from ln[unreacted α-dicarbonyl] versus time plots.

Enzyme Purifications. AK1 from chicken muscle was purified using a four-step protocol (*25*). A pH fractionation, a Zn acetate precipitation, an ammonium sulfate precipitation, and a gradient elution on phosphocellulose followed by gel filtration over Sephadex G-25 gave a homogeneous preparation of the enzyme. LDH from goat muscle was purified using a three-step protocol (*26*). A two-step ammonium sulfate precipitation and affinity chromatography over Sepharose Blue and then over Sepharose-oxamate gave a purified mixture of isozymes. The enzymes were assessed as being >90% pure on the basis of SDS−PAGE analysis (data not shown).

Enzyme Inactivations. All chemical modifications and enzyme inactivations were performed at 27 °C in the dark. All enzymes were desalted over Sephadex G-25 in Hepes or Bicine buffer and were adjusted to the requisite dilution to achieve 0.5 or 1 enzyme unit at the time of final assay. PGO was added to 5 mM, except against SOD in which case it was 40 mM. Aliquots withdrawn periodically were assayed for residual enzyme activities. Controls lacking PGO revealed that all enzymes were stable during the period of kinetic runs. Spontaneous inactivation, up to 15% during the kinetic run, was noted in the case of AK1 and PK at pH 9.0 and above. The k_{obs} values were corrected for spontaneous enzyme inactivations. The ln(remaining activity) versus time plots were linear till at least 80% inactivation for all enzymes except ADH, PK, and 3-PGK. All these enzymes are known to feature multiple modifiable arginines, thus explaining multiexponential inactivation curves (*27*−*29*). The initial linear regions of ln(remaining activity) versus time plots were used for k_{obs} calculation without any corrections. Thus, the k_{obs} in these enzymes represents the apparent modification rate of the fastest reactive arginine.

Enzyme Assays. All assays were carried out in a total volume of 3 mL at 27 °C and were initiated by the addition of 50 μL of an enzyme or enzyme/inhibitor solution, achieving a 60-fold dilution which effectively terminates the enzyme inactivation. The LDH, MDH, CK, HK, and AK1 assays were monitored at 340 nm by noting either the NAD(H) oxidation or NAD^+/NADP reduction. The LDH assay was carried out in 85 mM potassium phosphate (pH 7.5) with 0.2 mM NAD(H) and 2 mM sodium pyruvate (*30*). The MDH assay was carried out in 120 mM glycine buffer (pH 10.0) with 6.3 mM L-malate and 2.7 mM NAD^+ (*31*). The HK assay was carried out in 100 mM Tris-HCl buffer (pH 8.0) with 25 mM D-glucose, 10 mM ATP, 25 mM $MgCl_2$, 10 mM KCl, 2 mM NADH, 2.2 mM phosphoenol pyruvate, and 1 unit each of LDH and PK (*32*). The CK assay was carried out in 30 mM Hepes (pH 7.1) with 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 1.2 mM ADP, 20 mM glucose, 0.6 mM NADP, 20 mM creatine phosphate, and 2 units each of glucose-6-phosphate dehydrogenase and HK (*33*). The 3-PGK assay was carried out in 30 mM triethanolamine HCl buffer (pH 7.6) with 10 mM 3-phosphoglycerate, 1 mM $MgCl_2$, 40 mM ammonium sulfate, 5 mM ATP, 0.2 mM sodium EDTA, 0.2 mM NADH, and 8 units of gleceraldehyde-3-phosphate dehydrogenase (*34*). The AK1 assay was carried out in 50 mM Hepes-KOH buffer (pH 7.5) with 80 mM KCl, 1.4 mM MgCl, 0.35 mM PEP, 0.8 mM ATP, 2.3 mM AMP, 0.18 mM NAD(H), and 5 units each of LDH and PK (*35*). The SOD assay was carried out in 50 mM sodium carbonate (pH 10.2) with 0.1 mM EDTA and 0.3 mM epinephrine and was monitored at 480 for adrenochrome accumulation (*36*). The ADH assay was carried out in 20 mM glycine-NaOH buffer (pH 8.9) containing 0.1 M sodium pyrophosphate, 1 mg/mL BSA, 0.3 mg/mL glutathione, 0.5 M ethanol, and 2.5 mM NAD^+. The increase in absorbance at 340 nm due to NAD^+ reduction was monitored (*37*). The PK assay was carried out in 50 mM Hepes-KOH buffer (pH 7.5) containing 100 mM KCl, 2.5 mM PEP, 10 mM $MgSO_4$, 2 mM ADP, 0.2 mM NADH, 10 units of LDH, and 1 unit of PK (*37*).

Stoichiometry Measurements. The stoichiometry measurements were undertaken by incubating M_4-LDH (0.2 mg/mL), H_4-LDH (0.1 mg/mL), cytoplasmic MDH (0.4 mg/mL), and mitochondrial MDH (0.15 mg/mL) with [^{14}C]PGO (10 mM; the concentration measurement was based on an ϵ of 11 600 mol^{-1} cm^{-1} for PGO in water at 249 nm) in Hepes (50 mM) at pH 8.0 and by the measurement of remaining enzyme activity, as described in a preceding section, and the radioactivity incorporated into the protein, as described below, in a 20 μL aliquot sampled periodically. For radioactivity measurements, the aliquots were blotted on Whatmann 3 mm filter paper, which was soaked for 30 min in chilled 10% TCA and for 15 min in chilled 5% TCA, then rinsed with chilled 5% TCA and next with 95% ethanol, and finally oven-dried and counted in 5 mL of a scintillant made up of 0.4% PPO and 0.01% POPOP in toulene. The plots of residual enzyme activity versus the moles of PGO reacted per mole of protein (the protein concentration determination was by the Bradford method) gave the reaction stoichiometries. It was thus established that all the enzymes examined are with a reaction stoichiometry of 1 till up to at least 60% enzyme inactivation, which tends to increase to higher values thereafter.

pK_a Calculations. Of the 13 enzymes investigated, crystal structure coordinates of acceptable quality (a resolution of 2.7 Å or better) are available for the seven shown in Table 4. As far as 3-PGK is concerned, the enzyme in our experimental study is from porcine muscle while the available crystal structure coordinates are for the yeast enzyme.

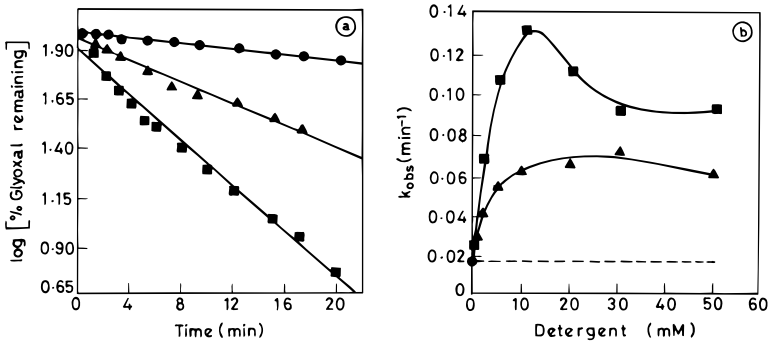


FIGURE 1: Detergent effects on OGn reactivity. OGn (10 mM) was reacted with DMAPGO (100 μM) at pH 9.0, in the absence (●) or presence of Triton X-100 (▲) or CTAB (■) (20 mM each in panel a and 0−50 mM each in panel b), and the decrease in DMAPGO concentration was monitored spectrophotometrically. Log(% DMAPGO unreacted) is plotted against time in panel a, and k_{obs} (min^{-1}) is plotted against detergent concentration in panel b. The dotted line in panel b refers to the k_{obs} in the absence of detergents.

The structure coordinates in the case of SOD and AK1 are for free enzymes, while in case of the others, as noted in Table 4, these are for either ternary complexes (M_4- and H_4-LDH) or binary complexes (both the MDH and 3-PGK) involving specific active sites ligands. The MDH structure coordinates are for homodimers, while the LDH structure coordinates are for homotetramers.

Electrostatic calculations were performed on a single protein subunit of each enzyme after stripping it of all ligands, including any crystallographic water molecules. Finite difference solutions of the Poisson−Boltzmann (FDPB) equation, implemented in the program FDCALC of J. Warwicker (*38*, *39*), were used, and the electrostatic field at the guanidinium group of particular arginines was calculated. The calculations take into account both the atomic partial charges, which are assigned from the GROMOS library (*40*), and the charges on ionizable groups corresponding to that expected at pH 7.0 with the pK_a values as follows: arginine (pK_a = 12.0) has +0.5 on Nη1 and Nη2, lysine (pK_a = 10.1) has +1.0 on Nξ, histidine (pK_a = 6.5) has +0.5 on Nδ1 and Nϵ2, aspartic acid (pK_a = 4.5) has −0.5 on Oδ1 and Oδ2, and glutamic acid (pK_a = 4.5) has −0.5 on Oϵ1 and Oϵ2. The explicit shape of a protein enters into consideration through the use of a probe with a 1.4 Å radius to demarcate the counterion and solvent accessible regions. The solvent is assigned the dielectric value 80, and the protein is assigned the dielectric value 3. A counterion response of 0.05 and a pH of 7.0 are used, the same as those used for most of our experimental determinations. The calculations are initiated with a 2.0 Å grid covering the protein and solvent, extending to zero potential boundary about 30 Å into the solvent, followed by a 0.5 Å grid focus run centered on the arginine guanidino group. The final electrostatic potential (millivolts) obtained at a specific GnH^+ group is converted into ΔpK_a by the formula (*39*) $\Delta pK_a = e\Delta\phi i/2.303K_BT$, where ϕi is the potential, K_B the Boltzmann constant, and T the temperature (298 K).

Analysis of Structures. The atomic environments of guanidino groups of arginines were analyzed with the help of homemade software which, when it is applied to a PDB file, outputs all the atoms within the specified radius of the target atom. In our results, the hydrogen bonds denote heavy atom distances of ≤3.5 Å. Sequence homology analyses in LDHs, MDHs, and AK enzymes were performed using FASTA (*41*).

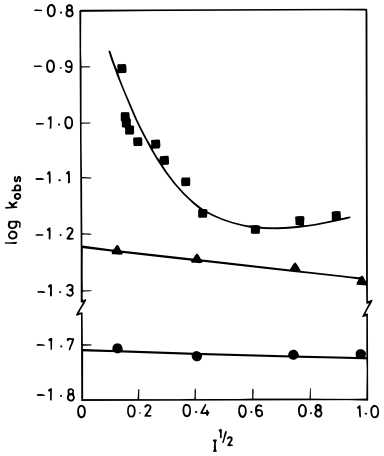


FIGURE 2: Ionic strength effects on OGn reactivity in the absence and presence of detergents. OGn (10 mM) in the absence (●) or presence of Triton X-100 (▲) or CTAB (■) (10 mM each) was reacted at pH 9.0 and the indicated ionic strength with DMAPGO (100 μM), and decrease in DMAPGO concentration was monitored spectrophotometrically. The log(k_{obs}) is plotted against the square root of the ionic strength ($I^{1/2}$).

RESULTS

ΔpK_a Measurements in Micellar Systems. OGn is the arginine side chain model used to assess GnH^+ for the effect of the microenvironment at the micelle−water interface. The results are summarized in Figures 1 −3 and in Table 1. Both CTAB and Triton X-100 are found to activate GnH^+ in a concentration-dependent manner (Figure 1a,b). The k_{obs} in water (0.016 min^{-1}) increases 4- and 8-fold (0.064 and 0.132 min^{-1}) in the presence of Triton X-100 and CTAB (20 mM each), respectively. The concentration-dependent effect of CTAB, but not that of Triton X-100, is biphasic. The reason is unclear; however, the maximal GnH^+ activation occurs at a detergent concentration (≈10 mM) that is much higher than the critical micellar concentration of both CTAB (0.9 mM) and Triton X-100 (2.0 mM) (*42*). Apparently, the detergent effects are a consequence of GnH^+ becoming micellarized, and for 10 mM OGn to be optimally micellarized, 10 mM CTAB and Triton X-100 seem to be required.

The results regarding the sensitivity of GnH^+ to ionic strength are summarized in Figure 2. OGn is more or less ionic strength insensitive in both water and Triton X-100, while its reactivity in CTAB diminishes progressively at increasing ionic strengths. Apparently, in Triton X-100, the GnH^+ is nonelectrostatically activated, while in CTAB, it is

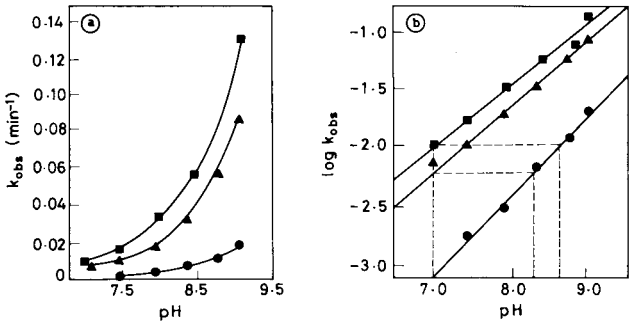


FIGURE 3: pH effects on OGn reactivity in the absence and presence of detergents. OGn (10 mM) in the absence (●) or presence of Triton X-100 (▲) or CTAB (■) (10 mM each) in Hepes (between pH 6.5 and 8.0) or Bicine (between pH 8.5 and 9.5) buffer (50 mM, ionic strength of 0.05) was reacted with DMAPGO/PGO (100 μM), and the decrease in DMAPGO/PGO concentration was monitored spectrophotometrically. k_{obs} and log(k_{obs}) are plotted against pH in panels a and b. The lines in panel b are linear least-squares lines.

Table 1: Slopes in pH Profile Plots, the ΔpK_a's Measured Using These Plots, and the ΔpK_a's Calculated Using PDB Files and the Protein Electrostatic Calculating Program FDPB

detergent or enzyme	slope	measured ΔpK_a	calculated ΔpK_a[a]
OGn	0.67	−	−
OGn−CTAB	0.52	−1.7	−
OGn−Triton X-100	0.58	−1.4	−
BGn	0.71	−	−
AK1 (porcine muscle)	0.38	−2.1	−1.5 (R97)
AK1 (chicken muscle)	0.38	−2.2	−
AK1 (rabbit muscle)	0.30	−2.3	−
HK (yeast)	0.43	−0.9	−
CK (rabbit muscle)	0.65	−2.4	−
3-PGK (porcine muscle)	0.73	−1.0	−
PK (rabbit muscle)	0.78	−1.1	−
LDH (porcine M_4)	0.60	−1.0	−2.0 (R171)
LDH (chicken muscle)	0.42	−1.3	−
LDH (goat muscle)	0.48	−0.9	−
LDH (porcine H_4)	0.57	−0.6	−0.5 (R171)
MDH (porcine mit)	0.69	−1.6	−1.5 (R152)
MDH (porcine cyt)	0.63	−0.7	−1.3 (R161)
ADH (yeast)	0.46	−0.8	−
SOD (erythrocyte)	0.34	+1.6	+1.7 (R141)

[a] The residue in parentheses is the arginine for which the pK_a is calculated.

at least partly electrostatically activated. At high ionic strengths, when the electrostatic effect in CTAB will be fully quenched, the GnH^+ continues to remain nearly as activated in CTAB as in Triton X-100. Thus, CTAB activates GnH^+ both electrostatically and nonelectrostatically, while the salt titration experiments make is possible for the effects of electrostatic and nonelectrostatic nature to be at least operationally distinguished.

The results regarding pH dependence of GnH^+ reactivity in water and in the micellar microenvironment are depicted in Figure 3. Since all modifications begin to titrate at pH ≈8.0 or lower (Figure 3a), the titrating species is unlikely to be GnH^+ since the expected pK_a of OGn in water is ≈13.0 (*43*). It is possible that the monocarbinolamine **Ia** (Scheme 1), being guanidinium ion with an electron-withdrawing substituent, is the species that titrates. The log k_{obs} versus pH plots (Figure 3b) are linear and have slopes with comparable magnitudes (see Table 1). A common species possibly titrates but with a different pK_a in water and at the water−micelle interface. To extract the pK_a change in GnH^+ on it being transferred from water to a water−micelle interface, extrapolations are performed on the pH profile plots as illustrated in Figure 3b. The extrapolations, by measuring the pH change to make the GnH^+ in water as reactive as the GnH^+ at the water−micelle interface, seek to indirectly measure the micelle-induced pK_a changes (i.e., ΔpK_a) on GnH^+ being transferred from water to the micellar microenvironment. From the extrapolations shown, it is noted that, when the GnH^+ in water is made as reactive as the GnH^+ in Triton X-100, the pH needs to be raised from 7.00 to 8.37 while, when it is made as reactive as the GnH^+ in CTAB, the pH needs to be raised from 7.00 to 8.74. Accordingly, the apparent ΔpK_a is −1.37 in Triton X-100 and −1.74 in CTAB (Table 1). From the salt titration experiment, we have already concluded that the ΔpK_a in Triton X-100 is of nonelectrostatic origin while the ΔpK_a in CTAB is of partly electrostatic and partly nonelectrostatic origin.

Table 2: *F* Values [k_{2app} in Enzyme Inactivation/k_{2app} in BGn Modification] at Specified pH and Ionic Strength Values

	pH 7.0		pH 8.0		pH 9.0	
enzyme	min[a]	max[a]	min	max	min	max
AK1 (porcine muscle)	29.2	13.7	17.5	6.6	7.5	6.6
AK1 (chicken muscle)	23.9	13.5	13.2	7.5	8.7	6.4
AK1 (rabbit muscle)	54.8	61.3	12.5	7.9	4.5	4.9
HK (yeast)	5.0	3.2	2.4	2.0	1.6	1.3
CK (rabbit muscle)	3.7	2.3	49.1	26.9	19.2	25.6
3-PGK (porcine muscle)	2.2	2.7	2.2	2.5	6.5	5.2
PK (porcine muscle)	2.7	9.2	3.7	8.3	2.0	7.0
LDH (porcine M_4)	22.4	0.7	4.8	1.4	3.6	2.1
LDH (chicken muscle)	18.2	2.2	7.8	2.0	4.1	1.3
LDH (goat muscle)	9.4	2.7	4.6	1.6	2.7	1.1
LDH (porcine H_4)	6.0	2.5	0.9	2.2	0.7	0.9
MDH (mit)	24.8	6.3	33.2	4.4	12.8	3.8
MDH (cyt)	2.8	2.3	3.1	2.1	2.8	2.0
ADH (yeast)	1.4	4.8	0.5	1.2	0.05	0.9
SOD (erythrocyte)	0.01	0.01	0.3	0.04	0.08	0.05

[a] Min and max correspond to the *F* values at zero and infinite ionic strengths, respectively, calculated using k_{2app}s corresponding to the intercepts (for zero ionic strength) and the assymptotes (for infinite ionic strength) to the *Y* axis in pH profile plots of the type shown in panels a and c of Figure 5.

ΔpK_a Measurements in Proteins. BGn is the arginine model used in this part of the study since it represents an arginine removed from a protein, along with its α-carbon but without the flanking amide units, and placed in water. Thus, in measurements of protein-mediated changes in arginine pK_a, BGn is the appropriate reference.

The k_{obs} (M^{-1}) values in enzyme inactivations are pseudo-first-order in enzyme concentrations, while the k_{obs} (M^{-1}) in BGn modification are pseudo-first-order in PGO concentrations. The k_{2app}'s (mol^{-1} min^{-1}), derived as k_{obs}/[PGO] in enzyme inactivations and as k_{obs}/[BGn] in BGn modification, are presented in Figures 4 and 5, either as such or as the logarithms. The minimum and maximum k_{2app}'s, defined as the k_{2app}'s at zero and infinite ionic strength, are derived as the intercepts of and asymptotes to the plots in Figures 5 and other similar plots at pH 8.0 and 9.0 (not shown). The values are presented in Table 2 as the ratios of k_{2app} in enzyme inactivation to the k_{2app} in BGn modification, which we designate as *F* values. The *F* values, denoting the degree of activation of an arginine relative to BGn, are noted to vary widely, being as low as 0.01 in the case of SOD and as high as ∼60 in the case of AK1. In fact, all the *F* values

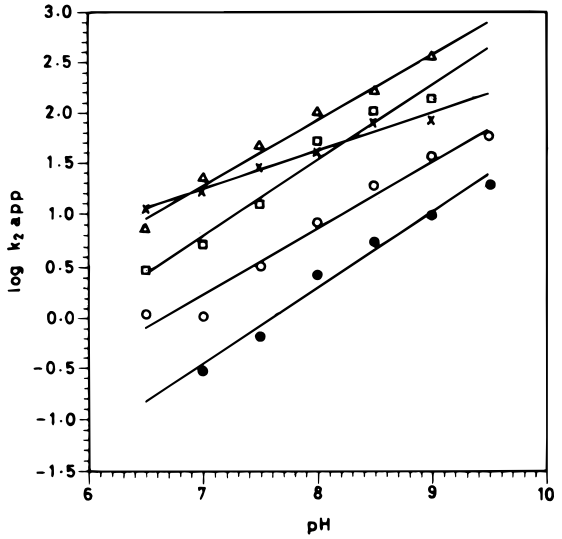


FIGURE 4: pH effects on arginine and BGn modification rate. Each enzyme (1 unit) was reacted with PGO (5 mM), and BGn (10−100 mM) was reacted with PGO (100 μM) in Hepes between pH 6.5 and 8.0) or Bicine (between pH 8.5 and 9.5) buffer (50 mM, ionic strength of 0.05), and the decrease in enzyme activity or PGO concentration, as required, was monitored as described in Experimental Procedures. Log(k_{2app}) values are plotted against pH. Each point is a mean of three independent determinations, and all lines are linear least-squares lines: LDH (M_4) (○), MDH (mit) (□), AK1 (×), CK (△), and BGn (●).

except those of SOD and ADH are noted to be greater than unity irrespective of the pH or ionic strength. Thus, with specific exceptions, all the modifiable arginines are "activated", although the magnitude of activation is appreciable in only about half of the arginines examined.

Representative log(k_{2app}) versus pH plots are shown in Figure 4. These and other such plots for extracting the ΔpK_a's are fitted with linear least-squares lines. All the line fits were satisfactory except for a comparatively larger scatter of data points for SOD due to an arginine with an exceptionally diminished reactivity. Also, an apparent deviation from linearity was observable for ADH, 3-PGK, and PK. Multiple arginines can be modified in these enzymes (*27*−*29*), making their PGO inactivations polyexponential. The slopes measured from pH profile plots, summarized in Table 1, are found to range from 0.30 in AK1 to 0.78 in PK, in contrast with the comparatively narrower range in the model reactions (0.53 for OGn in CTAB and 0.71 for BGn in water). Apparently, the pK_a's of titratable elements in proteins are pH sensitive. Meaningful comparisons between ΔpK_a's can thus only be made at a common reference pH. The ΔpK_a's at the reference pH 7.0, extracted exactly as in micellar experiments, are presented in Table 1. All the ΔpK_a's are negative with the solitary exception of that for SOD, implying in this case an increased pK_a relative to that of BGn. Some of the ΔpK_a's are even greater than the pK_a change in GnH^+ brought about in CTAB. The most diminished pK_a's are observed to be in CK and in the AK1s. The close match between the ΔpK_a's of three AK1s from different sources is noteworthy. The ΔpK_a's in the cases of MDHs and LDHs reflect isozyme specific variations. Among LDHs, the ΔpK_a is comparatively greater for the M_4 isozyme, while among MDHs, the ΔpK_a is comparatively greater for the mitochondrial isozyme.

The results of salt titration experiments are given in igure 5 and Table 2. The k_{2app} for BGn modification is ionic strength insensitive at every pH; thus, the observed sensitivities in protein experiments are due to effects of protein origin. From the results depicted in Figure 5a, the ionic strength effects at pH 7.0 are of an appreciable magnitude in some enzymes (M_4-LDH, mitochondrial MDH,

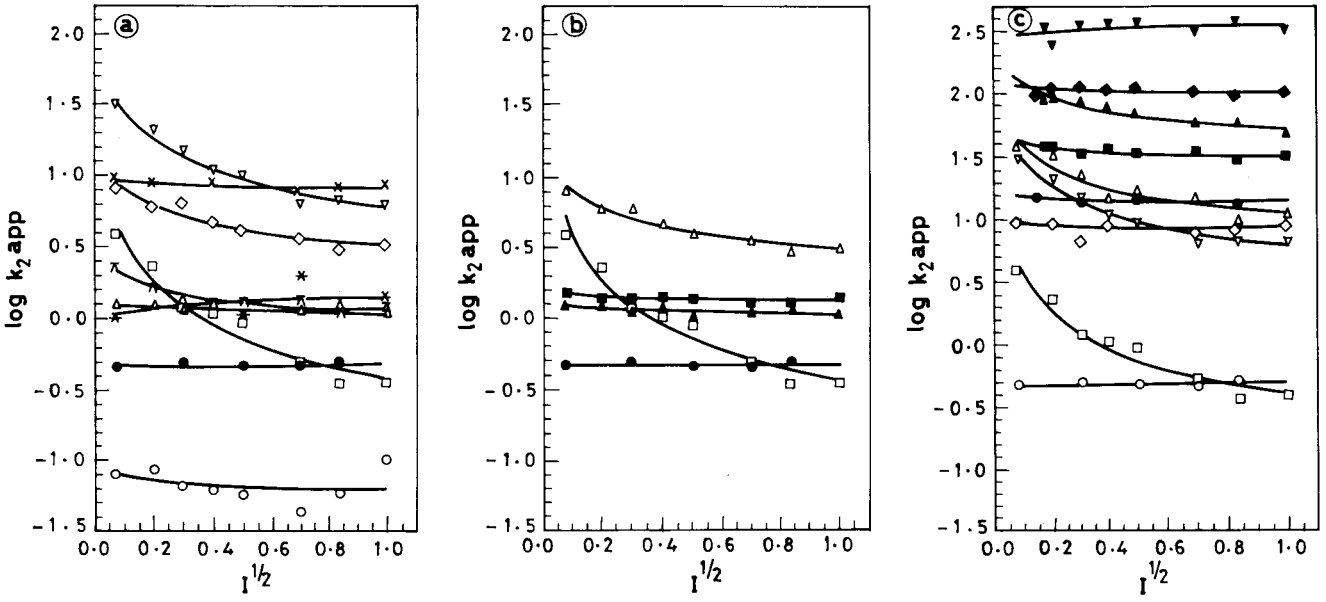


FIGURE 5: Ionic strength effect on arginine and BGn modification rate. Each enzyme (1 unit) was incubated with PGO (always 5 mM but 40 mM against SOD), and BGn (10−100 mM) was reacted with PGO (100 μM) in Hepes (pH 7.0) or Bicine (pH 9.0) buffer with a specified ionic strength; the decrease in enzyme activity or in PGO concentration, as required, was monitored as described in Experimental Procedures. Log(k_{2app}) values are plotted against the square root of the ionic strength ($I^{1/2}$). Each point is a mean of three independent determinations, while all lines are log−linear plots least-squares fits. (a) SOD (○), LDH (M_4) (□), MDH (cyt) (△), MDH (mit) (◇), HK (rotated k), 3-PGK (×), CK (▽), AK1 (*), and BGn (●); (b) isozyme-specific responses, LDH (M_4) (□) and MDH (mit) (△) (open symbols) and LDH (H_4) (■) and MDH (cyt) (▲) and BGn (●) (filled symbols); and (c) pH-dependent responses, AK1 (◇), CK (▽), MDH (mit) (△), LDH M_4 (□), and BGn (○) at pH 7.0 (open symbols) and at pH 9.0 (filled symbols).

Table 3: Functional and Structural Arginines with Diminished pK_a's[a]

enzyme	functional arginines[b]	structural arginines[c]	total
adenylate kinase	6 (6)	5 (0)	11 (6)
3-phosphoglycerate kinase	4 (1)	9 (1)	13 (2)
lactate dehydrogenase (M_4)	5 (4)	6 (1)	11 (5)
lactate dehydrogenase (H_4)	5 (5)	3 (0)	8 (5)
malate dehydrogenase (mit)	4 (4)	4 (0)	8 (4)
malate dehydrogenase (cyt)	6 (4)	4 (0)	10 (4)
superoxide dismutase	1 (0)	3 (0)	4 (0)
total	31 (24)	34 (2)	65 (26)

[a] The numbers refer to the number of arginines of a particular kind, while the numbers in parentheses refer to the number of arginines with diminished pK_a's. [b] All arginines in the active site clefts and at the subunit interface, irrespective of the roles. [c] All arginines except those defined as functional.

and CK) and negligible in others (AK1, 3-PGK, HK, and SOD). Thus, effects of electrostatic or nonelectrostatic nature are responsible for the arginines being activated. The electrostatic effect apparently is the largest in M_4-LDH, accounting for almost all of the arginine activation in this case, and somewhat smaller in mitochondrial MDH and CK, as their arginines continue to be at least partially activated even as the ionic strength effect saturates. The electrostatic contribution in arginine activation in AK1, 3-PGK, and HK apparently is negligible. Possibly the effects predominantly are in the nature of solvation effects.

The ionic strength sensitivities of LDHs and MDHs, compared in Figure 5b, are found to be isozyme specific. M_4-LDH and mitochondrial MDH, the isozymes with larger ΔpK_a's (Table 1), also are the isozymes that are more sensitive to ionic strength. Apparently, electrostatic effects activating arginines are larger in these isozymes than in H_4-LDH and cytoplasmic MDH.

Some noteworthy results from ionic strength variation experiments at different pH values are shown in Figure 5c. AK1, reflecting the ionic strength insensitivity at pH 7.0, is noted to be also ionic strength insensitive at pH 9.0. CK, LDH, and MDH, reflecting the ionic strength sensitivity at pH 7.0, are noted to display contrasting ionic strength sensitivities at pH 9.0. The ionic strength sensitivity in CK is almost completely abolished at pH 9.0; in LDH, it is diminished at pH 9.0, while in MDH, it is more or less unchanged at pH 9.0. Ostensibly, the electrostatic effect in CK at pH 7.0 is abolished completely at pH 9.0, in LDH at pH 7.0 is diminished at pH 9.0, and in MDH at pH 7.0 is more or less unchanged at pH 9.0. Thus, the electrostatic effects are either fully or partially pH titratable or untitratable in the pH range examined.

ΔpK_a Calculations in Proteins. Seven enzymes are analyzed computationally for the electrostatic fields in their arginines, using the protein structure coordinates excluding the heteroatoms in the PDB files shown in Table 4. The calculations are thus carried out with the proteins stripped of all ligands, including any metal ions or crystallographic water molecules. The LDH and MDH calculations are with the A subunits in the respective PDB files. The MDH A and B subunits have small conformational differences due to a lattice asymmetry (*44*), while the LDH subunits are identical (*45*).

As shown in Table 3, the 65 arginines covered in the study are grouped into two categories, "structural" and "functional", as follows. Any arginine within the active site cleft of an enzyme or in the interface between protein subunits, irrespective of its explicit role, is defined as a functional arginine. Invariably, the arginines are associated with ligands or with another protein subunit, but this is not always the case. For instance, there are functional arginines in AK1 and SOD active sites serving as substrate anchors that are unligated because the crystal structure is for the ligand free enzyme (*46*, *47*). Also, R65, R130, and R168 in the 3-PGK active site are by definition functional arginines but with no known roles in ligand binding. Any arginine not qualifying as functional on the locational consideration is defined in this study as a structural arginine. Invariably, the structural arginines have extensive H bonds within a protein and are thus unsuitable as ligand anchors. From Table 3 and Figures 6 and 7, a strong correlation is observed between the identity of an arginine as structural or functional and the electrostatic microenvironment of its GnH^+. Twenty-four of the 31 functional arginines are in an unfavorable electrostatic microenvironment capable of diminishing their pK_a's, while only two of the 34 structural arginines are in a microenvironment that is electrostatically unfavorable **(**Table 3 and

Table 4: Enzymes, Brookhaven PDB Codes, and Forms in the Crystal State, along with the PGO Modifiable Arginines versus the Total Number of Arginines

enzyme (PDB code)	form of the enzyme	PGO modifiable/ number of arginines	identity and reference of modifiable arginine
AK1 (3ADK)	free enzyme	1/11	1 (*54*) (R97)
HK (−)	−	1/18	1 (*64*) (NK)[a]
CK (−)	−	1/18	1 (*67*) (NK)[a]
3-PGK (3PGK)	ternary complex (ATP−PG)	2/13	2 (*29*) (NK)[a]
LDH (M_4) (9LDT)	ternary complex (NADH−oxamate)	1/11	1 (*57*, *58*) (R171)
LDH (H_4) (5LDH)	ternary complex (NAD−lactate)	1/9	1 (*58*, *65*) (R171)
MDH (mit) (1MLD)	binary complex (citrate)	1/8	1 (*58*) (R152)
MDH (cyt) (4MDH)	binary complex (NAD)	1/10	1 (*58*) (R161)
SOD (COB1)	free enzyme	1/4	1 (*66*) (R141)

[a] Each enzyme except 3-PGK has a single modifiable arginine; the identities of the modifiable arginines are not known (NK) in HK, CK, and 3-PGK.

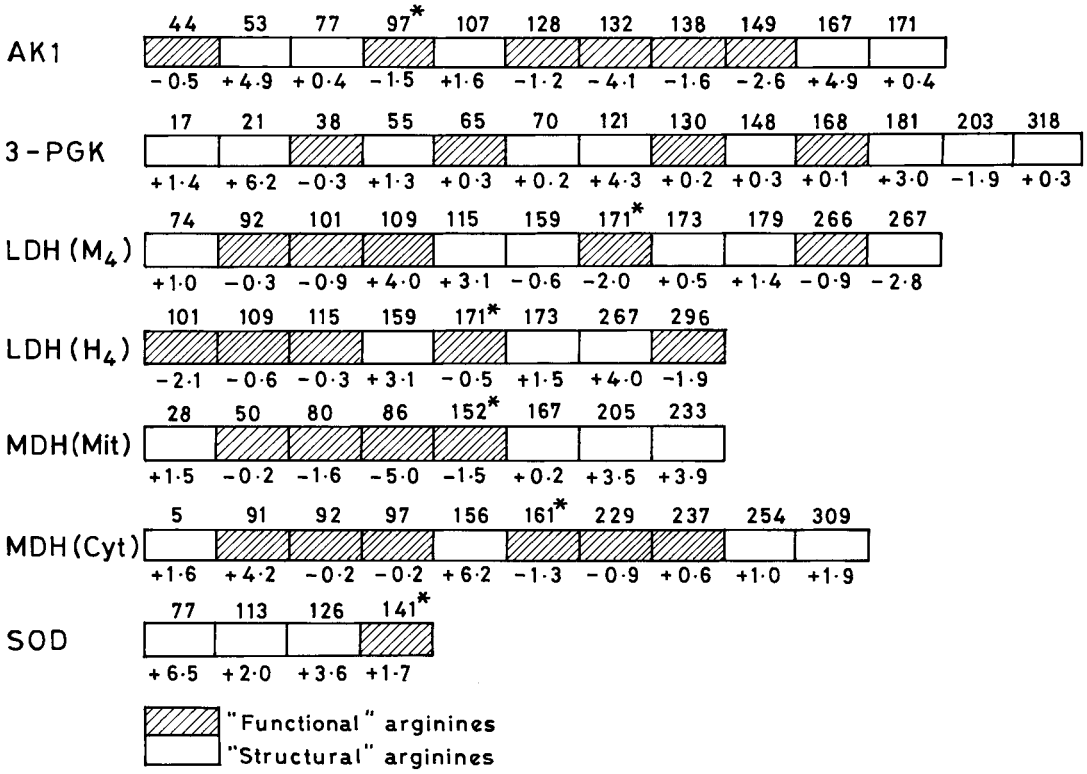


FIGURE 6: Relationship between arginine functional roles and guanidine ΔpK_a's. Functional arginines are all arginines within active site clefts and in the interface between protein subunits, while all other arginines are structural arginines. The number appearing above the boxes are the sequential arginine positions, while the numbers appearing below the boxes are the guanidine ΔpK_a's. The modifiable arginines are labeled with asterisks.

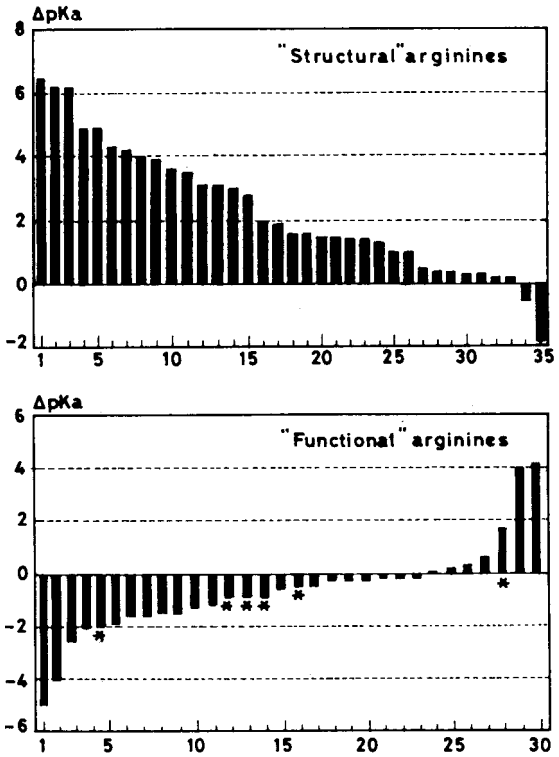


FIGURE 7: Structural and functional arginines in decreasing order of ΔpK_a's. Two of the structural arginines have negative ΔpK_a's, while 24 of the 31 functional arginines have negative ΔpK_a's. The modifiable arginines are labeled with asterisks.

Figure 7). Thus, the structural arginines have an average ΔpK_a of 2.4, while the functional arginines have an average pK_a of −0.9. The exceptional structural arginines in an unfavorable electrostatic microenvironment, and thus with diminished pK_a's, are noted in Figure 6 to be R203 in 3-PGK and R159 in M_4-LDH, while the exceptional functional arginines in an electrostatic microenvironment capable of enhancing their pK_a's are noted to be R141 in SOD, R109 in M_4–LDH, R91 and R237 in cytoplasmic MDH, and R65, R130, and R168 in 3-PGK.

The calculated ΔpK_a's of modifiable arginines in Table 1 are found to be reasonably consistent with the measured ΔpK_a's. However, we also note in Figures 6 and 7 that not every modifiable arginine has a diminished pK_a and that the modifiable arginine pK_a's are not necessarily the most diminished pK_a's. A number of issues, including arginine identities, the enzyme forms in crystal states, and assumptions and approximations inherent to the FDPB approach (*38*), are important in interpreting the results, which are taken up in the Discussion.

Local Effects on ΔpK_a's. The pK_a calculations included the effects of all charge-bearing atoms in a protein irrespective of their locations. There are reasons to believe, however, that the pK_a's of especially structural arginines could be primarily influenced by the atoms directly H-bonded to their GnH^+s or in close proximity to them. To test this premise, the GnH^+s were analyzed with respect to their atomic atmospheres, i.e., for the atoms within their solvation spheres, which in this study we define as three overlapping spheres with radii of 5 Å each centered on the nitrogens. Consistent with the observations of Singh and Thornton (*48*) in 52 protein PDB structures, the analysis revealed the remarkable propensity of the GnH^+s to be preferentially surrounded by oxygens invariably within H-bond distances. That the oxygens, always with a partial or formal negative charge,

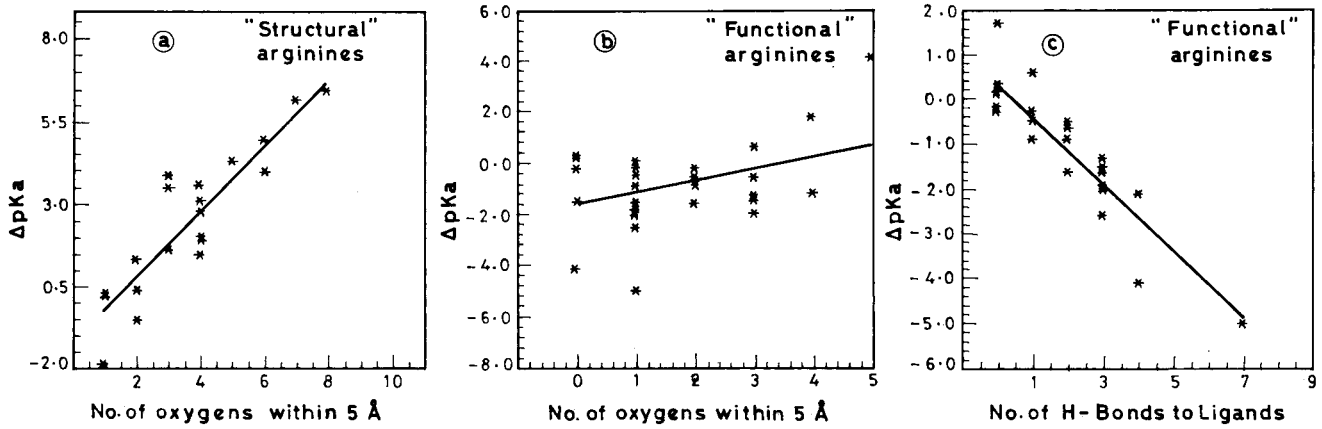


FIGURE 8: Relationship between arginine ΔpK_a's and the number of oxygens within the guanidine solvation sphere. (a) The ΔpK_a's of structural arginines vs the number of oxygens in their solvation spheres. (b) The ΔpK_a's of functional arginines vs the number of oxygens in their solvation spheres. (c) The ΔpK_a's of functional arginines vs the number of H bonds between their guanidines and the ligands.

could be the principal source of the electrostatic effect operating on structural arginines is borne out by the plot in Figure 8a. According to the plot, the arginine ΔpK_a's correlate strongly with the number of oxygens within 5 Å of their GnH^+s. Actually, the majority of oxygens are within H-bonding distance (3.5 Å), and their numbers correlate equally strongly with the observed ΔpK_a's (data not shown). The implication is that the electrostatic effects on structural arginines could be largely of local origin, possibly reflecting the structural role of the GnH^+ due to the degree of H bonding within a protein. In fact, it could be expected that a GnH^+ in an electrostatically unfavorable microenvironment would be unfavorable for protein conformation stability. That actually only two such GnH^+s are found among the 34 structural arginines is thus a point worth noting. In each of these arginines, we find within the solvation sphere of GnH^+ positively charged atoms or electrostatically neutral methylenes. Thus, R203 in 3-PGK is with NE of K196 (3.9 Å), NG of N364 (3.3 Å), and CG of T265 (3.3 Å) while R159 in M_4-LDH is with CG of P156 (3.2 Å), CG of P132 (3.4 Å), and CD of F155 (3.9 Å) in the solvation sphere of GnH^+, suggesting that the unfavorable effect diminishing the arginine pK_a could at least partly be of local origin.

Because of their roles, the GnH^+s of functional arginines are expected to be relatively free from atomic neighbors from within a protein. Although this is generally true, it is also found that the comparatively fewer protein atoms surrounding the arginines continue to be predominantly oxygens invariably H-bonded to the GnH^+s. However, as could be expected from the average electrostatic microenvironment of these GnH^+s, the number of oxygens does not correlate with the arginine ΔpK_a's as noted in Figure 8b. The electrostatic effects on functional arginines appear thus to be largely of nonlocal origin, although some of the GnH^+s do have atoms within their solvation spheres that could at least partly account for the electrostatic effects found to be operating.

Whatever their structural origins, the electrostatic effects on functional arginines evoke interest in knowing their functional significance. From the plot in Figure 8c, we note that the arginine ΔpK_a's correlate strongly with the number of H bonds between their GnH^+s and the ligands. Several specific examples of the correlation between the GnH^+ role and the unfavorable change in arginine pK_a are worth noting. Two sulfate ions in the AK1 active site are noted to form four H bonds with R132 and three H bonds each with R138 and R149 and are close enough (6.3 Å) to impose an electrostatic effect on R128. The ΔpK_a's of the arginines are noted to correlate strongly with the GnH^+ roles in sulfate binding. R86 in mitochondrial MDH forms seven H bonds with the active site citrate and happens to be the arginine with the most diminished pK_a in this enzyme. R161 in cytoplasmic MDH forms two H bonds with a sulfate and one H bond with the D58 carboxylate in another protein subunit and is the arginine with the most diminished pK_a in this enzyme. Most notably, R97 in AK1 although free from any ligand in the protein in the crystal state actually is the anchor for AMP (*49*). Thus, the arginine roles in anion binding seem to be electrostatically predetermined.

DISCUSSION

The catalytic roles of amino acid functionalities in enzyme active sites are strongly context-dependent. Undoubtedly, microenvironmental effects are involved and need to be investigated in bridging the gulf between the knowledge of structure and the understanding of the function of an enzyme. Often, an arginine is the critical residue, and the microenvironmental effects on its role need to be characterized. In light of the inaccessible nature of the arginine pK_a, we became interested in the phenomenon of activated arginines (*10*). In particular, we were guided by the notion that arginines could be activated by pK_a diminutions mediated by the microenvironmental effects in the so-called anion recognition centers (*10*, *11*). The broad consensus of results in this study is that the phenomenology of arginine activation probably has been correctly anticipated, and that the activated arginines indeed could be valuable probes in studying enzymes for active site effects that could be catalytically critical.

The change in an ionizable group pK_a from the intrinsic pK_a in water is the function of the difference free energy of the group in water and the group in a macromolecule. Both Triton X-100 and CTAB, providing a microenvironment that is unfavorable for protonated GnH^+, will diminish its pK_a. That the effects could activate arginines is borne out in the micellar simulation experiments presented here. Thus, it has been possible to measure the protein-mediated pK_a changes in GnH^+ from the degree of arginine activations, and for

the underlying activating effects to be probed on the basis of the responsiveness to pH and salt strength. The good match between the measured and calculated ΔpK_a's (Table 1) is in affirmation of the link between pK_a changes and the degree of arginine activation. However, several uncertainties remain with regard to the actual magnitudes of the ΔpK_a's. Although numerous activated arginines are reported, few are unequivocally identified because of the generally poor modification selectivities and the unstable nature of the modification products complicating the isolation and sequencing of labeled peptides (*12*). The modifiable arginines are well-identified in AK1 and SOD, reasonably certain in LDHs and MDHs, and essentially unknown in CK, ADH, 3-PGK, and PK (see Table 4). The other uncertainty in ΔpK_a estimates concerns their susceptibilities to the heterogeneous dielectric at the protein−water interface and the conformational effects due to protein thermal fluctuations and the effects of ligand binding. Both LDHs and MDHs in the crystal state have active site ligands; hence, their loop domains are in closed conformations (*44*, *45*, *50*, *51*), and as a result, the arginine microenvironments might be different in the crystal state and in solution. Although free from any specific active site ligands, the calculated ΔpK_a's in AK1 and SOD neither validate the measured ΔpK_a's nor explain the selective arginine modifications. R132, R138, and R149 in AK1 have more diminished pK_as than the chemically modifiable R97. However, sulfate ions in the enzyme active site could be responsible for the arginine microenvironment in the crystal state and hence for the calculated pK_a's. The arginine microenvironment in the free enzyme in the solution state needs to be known for the modification selectivity of R97 in AK1 to be adequately explained. Also, there are the metal ions Co^{2+} and Cu^{2+} in the SOD active site which could influence the R141 pK_a, calling into question the match between measured and calculated ΔpK_a's observed in this case. Thus, protein conformational effects and assumptions and approximations inherent to FDPB approach, threadbare in the literature (*38*, *52*, *53*), need to be considered in judging the pK_a comparisons we are attempting to make in this study. In summary, although arginines could be activated by microenvironmental effects that diminish the pK_a's of their guanidine groups, the ΔpK_a's we have obtained kinetically need to be validated, by unequivocally identifying the arginines and by addressing the uncertainties and approximations inherent to the FDPB approach.

The calculated pK_a changes are found to correlate strongly with the general arginine roles as structural or functional elements. The analysis of the atomic atmosphere of the GnH^+s establishes that with structural arginines the operative electrostatic effects are of largely local origin, due to the oxygens H-bonded to the GnH^+ on account of their primarily structural roles. With functional arginines, on the other hand, the GnH^+s are found to occur in an electrostatic microenvironment that may be unfavorable structurally but is favorable functionally, capable of reinforcing the argnine−anion interactions, and possibly causing some of the arginines to become chemically activated. The broad conclusion thus is that while the stereoelectronic attributes of GnH^+ may be favorable for its role as the anion anchor, equally important are the microenvironmental effects that can reinforce the GnH^+−anion interactions.

With these broad generalizations, we may now focus attention on the relationship between the activation and the functional roles of arginine as a catalytically critical element.

AK1. The single PGO modifiable arginine in porcine AK1, identified by Berghauser and Shirmer on the basis of peptide mapping, is R97 (*54*). The single PGO modifiable arginine in *Escherichia coli* AK, identified by Reinstein et al. (*55*) on the basis of sequence analysis of the [^{14}C]PGO-modified enzyme, is R88. R97 in porcine AK1 and R88 in *E. coli* AK are homologous arginines. R88 in the crystal structure of *E. coli* AK, complexed with Ap_5A, has a calculated ΔpK_a of −5.9 and happens to be the arginine with the most diminished pK_a in the enzyme. On the other hand, three active site arginines in porcine AK1 have pK_a's more diminished than those of R97. R97 in the crystal form of porcine AK1, however, is free from ligands, while R88 in *E. coli* AK is in direct contact with Ap_5A. R97 in porcine AK1, at the bottom of the active site cleft, is stereochemically "accessible" for both substrate binding and chemical modification. The effect of nonelectrostatic nature found to be responsible for the arginine activation could have a direct bearing on the 1−2 kcal mol^{-1} in AMP binding and the 3.5 kcal mol^{-1} in transition state stabilization that R97 contributes according to the results with site specific AK1 mutants (*49*).

LDH and MDH. At least three equivalent arginines perform analogous roles in LDHs and MDHs (*56*, *57*): one is the substrate anchor, another one is the coenzyme anchor, while the third one seems to contribute in hydride transfer due to the polarization of a substrate carbonyl (*51*). The arginines that can be modified by PGO appear to be, on the basis of protection experiments, R171 in LDHs, R161 in mitochondrial MDH, and R152 in cytoplasmic MDH (*44*, *57*, *58*). The arginines are functionally homologous, serving as the substrate anchors. In LDH, the arginine is critical for substrate binding since the K_m for pyruvate in a lysine mutant (R171K) is unfavorable by 5.5 kcal mol^{-1} (*59*). A mutant with tryptophan replacing R171 (R171W) is found to be catalytically active (*60*), and it is possible that the electrostatic effect observed at R171 anchors the anionic ligand in the R171W mutant. The stereospecificity in pyruvate reduction by LDH has been attributed to a positive electrostatic field in LDH at R171. Learau and Anderson (*61*) suggest that the field is from a helix dipole; however, the observation of its pH dependence in this study suggests that the field could originate from ionizable residues. The isozyme specificity of the field, in both LDHs and MDHs, is of interest for its possible role in the isozyme preferences for either NAD (H) or NAD^+, because the coenzymes have differences in their electrostatic attributes.

SOD. SOD is known to be associated with an electrostatic field that attracts and guides its negatively charged substrate toward the active site, accelerating the reaction beyond the limit set by the diffusional barrier (*62*). Surprisingly, we find that the enzyme may be devoid of a positive electrostatic field within its active site, despite the occurrence of the metal ions Cu^{2+} and Zn^{2+} within the active site (*47*).

3-PGK. The identities of the two PGO modifiable arginines in the porcine muscle and yeast 3-PGKs are unknown (*29*). Our calculations with yeast 3-PGK reveal two arginines that have diminished pK_a's. R38 (ΔpK_a = −0.3) is catalytically critical and, according to the crystal structure data, participates in substrate binding (*34*). There

is an anion activation site in 3-PGK, which is suggested to reside in a patch of basic residues consisting of H62, H167, H170, R21, R38, R65, and R168 (*63*). We find that none of the basic patch residues, except R38, has a diminished pK_a. Away from both the basic patch region and the active site, R203 has a diminished pK_a and an atomic atmosphere that could favor its anion interaction. Does this arginine participate in the phenomenon of anion activation, and does it also happen to be the chemically modifiable arginine in 3-PGK? These are interesting questions for future studies.

Conclusion. In conclusion, we have been able to establish that (i) microenvironmental effects mediating anion recognition indeed could be responsible for arginine activation, (ii) activated arginines can be the probes for unmasking the operative effects in an anion recognition center, and (iii) diminished local polarity and enhanced positive electrostatic potential appear to be effects mediating the arginine role as a catalytically critical residue.

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