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Specific Arginine Modification at the Phosphatase Site of Muscle Carbonic Anhydrase†

Linda M. Pullan‡ and Ernst A. Noltmann*

Department of Biochemistry and Division of Biomedical Sciences, University of California, Riverside, California 92521
Received May 1, 1984

ABSTRACT: Mammalian carbonic anhydrase III has previously been shown to catalyze the hydrolysis of *p*-nitrophenyl phosphate in addition to possessing the conventional CO_2 hydratase and *p*-nitrophenylacetate esterase activities. Modification of pig muscle carbonic anhydrase III with the arginine reagent phenylglyoxal yielded two clearly distinctive results. Reaction of the enzyme with phenylglyoxal at concentrations equivalent to those of the enzyme yielded stoichiometric inactivation titration of the enzyme's phosphatase activity, approaching 100% loss of activity with the simultaneous modification of one arginine residue, the latter based on a 1:1 reaction of phenylglyoxal with arginine. At this low ratio of phenylglyoxal to enzyme, neither the CO_2 hydratase activity nor the acetate esterase activity was affected. When the modification was performed with a significant excess of phenylglyoxal, CO_2 hydratase and acetate esterase activities were diminished as well. That loss of activity was accompanied by the incorporation of an additional half dozen phenylglyoxals and, presumably, the modification of an equal number of arginine residues. The data in their entirety are interpreted to show (a) that the *p*-nitrophenylphosphatase activity is a unique property of carbonic anhydrase III and (b) that excessive amounts of the arginine-modifying reagent lead to unspecific structural changes of the enzyme as a result of which *all* of its enzymatic activities are inactivated.

Begining with the serendipitous discovery in our laboratory (Koester et al., 1977a,b) and with similar findings by Holmes (1977) and by Tashian's group (Tashian et al., 1978), mammalian muscle carbonic anhydrase is now accepted as a new enzyme species (CA III) that is different and distinguishable from the extensively researched carbonic anhydrases I and II originally found in erythrocytes. In fact, many of the research groups recognized for their work on carbonic anhydrases I and II have now also turned to studies on this third carbonic anhydrase as exemplified by the topics of a recent conference on the biology and chemistry of the carbonic anhydrases sponsored by the New York Academy of Sciences (Chegwidden et al., 1984).

Prior to our identification as carbonic anhydrase III (Koester et al., 1977a,b; Register et al., 1978) of what was then "basic muscle protein", we conducted numerous experiments designed to screen for a biological function of the unknown protein which at that time had been characterized only by its chemical and physical properties. Only the testing for acid phosphatase activity with the substrate *p*-nitrophenyl phosphate gave positive results, so thought was given initially to phosphatase activity being *the* primary function of basic muscle protein. Information on the phosphatase activity and its characteristics vs. those of the classical CO_2 hydratase and acetate esterase activities has since been reported (Koester, 1979; Koester et al., 1981; Pullan & Noltmann, 1984).

The possible location of a phosphatase catalytic site on muscle carbonic anhydrase suggested chemical modification of the enzyme by reagents known to interact with arginine residues, the potential phosphate binding sites. This approach would be especially valuable if it should prove possible to abolish the phosphatase activity without interfering with either the CO_2 hydratase or the acetate esterase functions. The present paper demonstrates that we were indeed successful in

† This work was supported in part by National Science Foundation Research Grant PCM 78-16095 and by U.S. Public Health Service Biomedical Research Support Grant RR 05816. The investigations described in this paper are from a dissertation submitted by L.M.P. to the Graduate Division of the University of California, Riverside, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
‡ Present address: Monsanto Health Care Division, St. Louis, MO 63166.

0006-2960/85/0424-0635$01.50/0

modifying the phosphatase site of carbonic anhydrase III with phenylglyoxal under conditions that preserved its other catalytic functions. A preliminary report of part of this work has been given previously (Pullan & Noltmann, 1984).

Experimental Procedures

Materials

Carbonic anhydrase III was isolated from pig muscle by the method reported previously (Register et al., 1978). All of the materials not specified below were of reagent grade and were used without further purification. Doubly distilled deionized water was used throughout. Special reagents purchased include *p*-nitrophenyl acetate and *p*-nitrophenyl phosphate from Sigma, both containing less than 0.1% free *p*-nitrophenol as determined by absorbance readings at 405 nm. Sodium diethylmalonate was obtained from Mallinckrodt, and acetazolamide was purchased as Diamox from Lederle Labs. Phenylglyoxal (97%), purchased from Aldrich, was recrystallized 3 times from water prior to use. Butanedione from Sigma was used without further treatment. [^{14}C]Phenylglyoxal (32 mCi/mmol) was purchased from Research Products Incorporated.

Methods

CO_2 Hydratase Assays. Carbonic anhydrase CO_2 hydratase activity was determined in the direction of the hydration reaction by modification of the Wilbur–Anderson colorimetric method (Wilbur & Anderson, 1946) introduced by Rickli et al. (1948). Sodium barbital buffer (pH 8.2) containing 0.025% bromthymol blue was titrated to its colorimetric end point with a standardized solution of HCl to determine the concentration of protons required for the assay color change. The substrate, CO_2-saturated water, was prepared and maintained by continuously bubbling the gas sublimed from dry ice through doubly distilled water. From 0.04 to 0.4 μM enzyme was assayed in a mixture of 0.5 mL of sodium barbital and 0.5 mL of CO_2-saturated water, with the substrate added last to initiate the reaction. The uncatalyzed rate was obtained by adding an equivalent volume of buffer (and inhibitor, if present) without enzyme into the assay mixture. Enzyme activity units are defined as micromoles of H^+ per liter per second after subtraction of the corresponding uncatalyzed rate calculated in the same manner. Enzyme concentrations were determined by measurement of the absorbance at 280 nm with use of an absorbance coefficient ($E^{1\%}_{1cm}$) of 23.2 for the pig muscle isoenzyme. Turnover numbers are expressed according to Maren (Maren et al., 1960), with division of the rate in enzyme units by the micromolar enzyme concentration. All CO_2 hydratase assays were carried out in an ice bath.

Esterase Assays. The *p*-nitrophenylacetate esterase activity was determined by the method of Armstrong et al. (1966). A recording spectrophotometer was employed at the isosbestic wavelength of 348 nm for *p*-nitrophenol and the *p*-nitrophenoxide ion. The extinction coefficient corrected for the slight absorbance of the acetate ester was 5.0×10^3 M^{-1} cm^{-1} at pH 7.2 and 30 °C. The assay was performed with 0.9 mL of 33.3 mM sodium diethylmalonate and 1 mM *p*-nitrophenyl acetate with the uncatalyzed rate being recorded prior to addition of the enzyme. The sodium diethylmalonate buffer was chosen for its minimal contribution to the nonenzymatic rate (Pocker & Meany, 1965).

Phosphatase Assays. The *p*-nitrophenylphosphatase activity was measured by a quenched spectrophotometric assay previously described (Koester et al., 1981). Assays were performed at 30 °C in 0.9 mL of 0.1 M sodium succinate, pH 5.3, and 5 mM *p*-nitrophenyl phosphatase, with enzyme added to initiate hydrolysis. The concentration of the *p*-nitrophenoxide ion formed by addition of 0.2 mL of assay sample to 0.8 mL of 2 N NaOH was determined spectrophotometrically with use of an extinction coefficient of 1.85×10^4 M^{-1} cm^{-1}. Aliquots were removed at four time intervals and the concentrations of the *p*-nitrophenoxide ion determined so as to yield a rate of phenoxide ion released with time. Uncatalyzed rates of hydrolysis were determined in an analogous manner, and the enzymatic rate was then corrected.

Arginine Modification. For modification with phenylglyoxal or butanedione, a reaction mixture consisting of muscle carbonic anhydrase (usually at concentrations near 280 μM) and 3 times recrystallized phenylglyoxal (2–30-fold molar excess) was incubated with 50 mM sodium diethylmalonate, pH 7.0, in capped and foil-wrapped tubes kept in a water bath at 30 °C. Aliquots were withdrawn periodically, and the reaction was quenched by 3-fold dilution into ice-cold 50 mM sodium diethylmalonate, pH 7.0. Controls of enzyme alone, buffer alone, and the modifier alone were always separately measured. In addition, the inactivation was usually performed on duplicate or triplicate samples. Enzyme activities were measured by pipetting these samples into the appropriate assay mixture for the three catalyzed reactions of muscle carbonic anhydrase. Pseudo-first-order rate constants were obtained by multiplying the slopes of the semilogarithmic plots by 2.303. For modification reactions with 2,3-butanedione, the experiments were performed in 50 mM sodium borate buffer, pH 8.5, with the borate expected to electrostatically stabilize the enzyme complex with the modifier (Yankeelov, 1970).

The pH dependence of phenylglyoxal modification of the phosphatase activity of carbonic anhydrase III was determined by reaction in 50 mM sodium succinate at pH 5.5 and 6.0, in sodium diethylmalonate at pH 7.0 and 7.5, and in *N*,*N*-bis(2-hydroxyethyl)glycine (Bicine) at pH 8.0 and 9.0. The extent of inactivation was monitored as described above.

To determine the stoichiometry of inactivation, the enzyme was reacted with radioactively labeled phenylglyoxal (32 mCi/mmol) in a manner identical with that described above. The extent of incorporation of [^{14}C]phenylglyoxal was quantitated by scintillation counting after dialysis of the samples against four changes of 2 L each of ice-cold 50 mM sodium diethylmalonate, pH 7.0. The concentration of enzyme in each dialyzed sample was determined spectrophotometrically. The reliability of dialysis as a method to remove excess reagent was verified by assays of the samples before and after dialysis under conditions where no differences in specific activities were detected.

Results

Phosphatase Inactivation. Inactivation of the phosphatase activity by low levels of phenylglyoxal could be obtained with no loss of the hydratase or esterase activities. The pseudo-first-order loss of phosphatase activity was observed at concentrations of phenylglyoxal as low as the lowest concentration of the enzyme. For the conditions of the experiment presented in Figure 1 (268 μM pig muscle carbonic anhydrase, 1.2 mM phenylglyoxal, and 50 mM sodium diethylmalonate, pH 7.0, 30 °C), the pseudo-first-order rate constant is 0.48 h^{-1}. The esterase activity of phenylglyoxal-treated enzyme was not affected. The time for 50% loss of phosphatase activity was linearly proportional to the reciprocal of the concentration of phenylglyoxal (Figure 2) with a second-order rate constant of about 0.05 M^{-1} min^{-1}.

When the rate of inactivation by phenylglyoxal was studied as a function of pH, inactivation was found to occur at an increasing rate between pH 6 and 9, i.e., within the range of

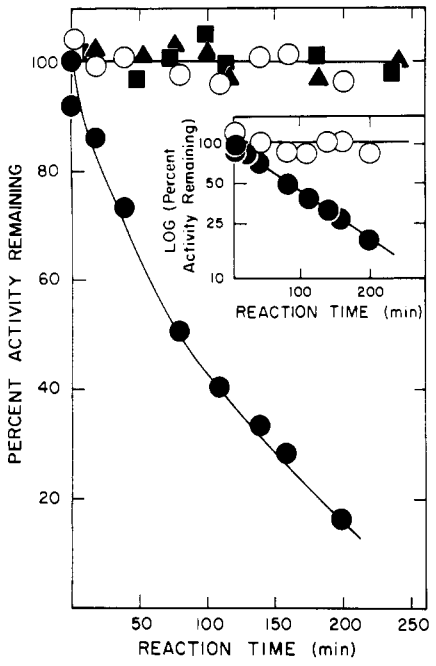

FIGURE 1: Modification of the *p*-nitrophenyl phosphatase activity of pig muscle carbonic anhydrase III by phenylglyoxal. Reaction conditions: 268 μM enzyme, 1.2 mM phenylglyoxal, and 50 mM sodium diethylmalonate, pH 7.0, 30 °C; (●) Phosphatase activity (▲), CO_2 hydratase activity; (■) p-nitrophenylacetate esterase activity, all in the presence of 1.2 mM phenylglyoxal; (○) control of phosphatase activity without phenylglyoxal. Inset: Semilogarithmic presentation of the inactivation of phosphatase activity.

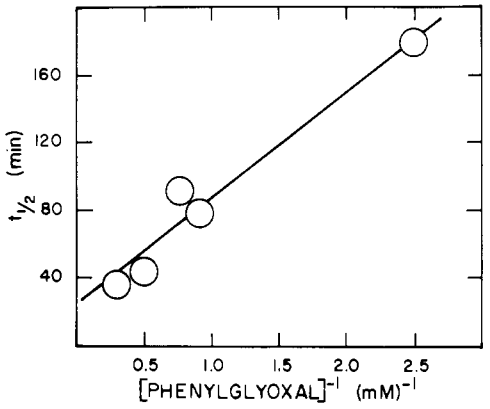

FIGURE 2: Effect of phenylglyoxal concentration on the time required for 50% inactivation of the *p*-nitrophenyl phosphatase activity of pig muscle carbonic anhydrase III. Reaction conditions: 280 μM enzyme and 50 mM sodium diethylmalonate, pH 7.0, 30 °C; phenylglyoxal concentrations are as indicated on the abscissa.

enzyme stability (data not shown). A true pH optimum could not be determined due to the contribution of enzyme instability to the inactivation of the enzyme at higher pH. Experiments were generally performed at pH 7 for maximal reproducibility, selectivity for arginine (Takahashi, 1977a,b), and stability of the covalent product.

Figure 3 shows the correlation of the level of phosphatase inactivation and the stoichiometry of incorporation of radioactively labeled phenylglyoxal in a demonstration of the specificity of the inactivation reaction of the muscle isoenzyme. The incorporation of [^{14}C]phenylglyoxal was directly proportional to the loss of enzymatic activity until about 75% of the phosphatase activity was lost, with very close to one phenylglyoxal incorporated at an extrapolated 100% loss of phosphatase activity. The rate of inactivation decreases after about 75% inactivation as would be expected from depletion of the reactants as well as from possible nonspecific reactions

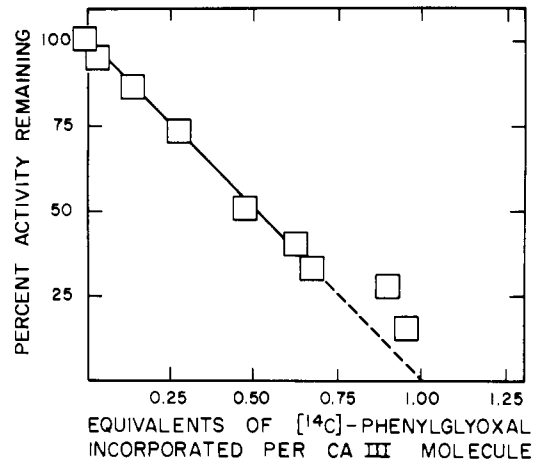

FIGURE 3: Inactivation titration of pig muscle carbonic anhydrase III with [^{14}C]phenylglyoxal. Reaction conditions are as described for Figure 1, with [^{14}C]phenylglyoxal.

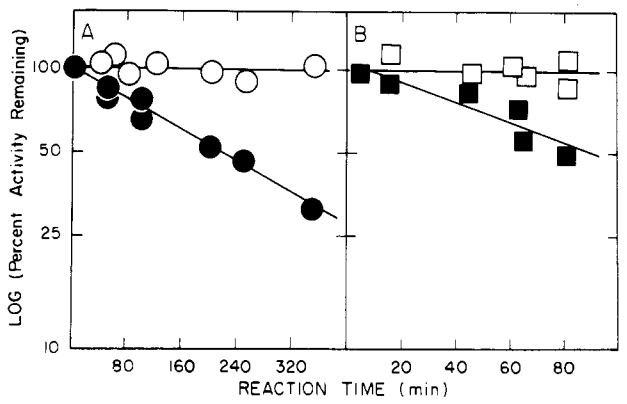

FIGURE 4: Semilogarithmic plots of the loss of CO_2 hydratase activity (A) and *p*-nitrophenylacetate esterase activity (B) following treatment of pig muscle carbonic anhydrase III with phenylglyoxal. (A) Conditions for inactivation of the CO_2 hydratase activity: (●) 280 μM enzyme, 13 mM phenylglyoxal, 50 mM sodium diethylmalonate, pH 7.0, 30 °C; (○) control without phenylglyoxal. (B) Conditions for inactivation of the *p*-nitrophenylacetate esterase activity: (■) 280 μM enzyme, 28.6 mM phenylglyoxal, and 50 mM sodium diethylmalonate, pH 7.0, 30 °C; (□) control without phenylglyoxal.

of phenylglyoxal. Cysteine was found not to be involved in the reaction with phenylglyoxal in that there was no difference in titration of the enzyme with *p*-mercuribenzoate before and after modification of the enzyme by phenylglyoxal (data not shown).

Hydratase and Esterase Inactivation. The CO_2 hydratase and esterase activities of pig and rabbit muscle carbonic anhydrase III could also be inactivated by modification with phenylglyoxal, but only at phenylglyoxal concentrations significantly higher than that required for phosphatase inactivation. Each activity was lost in a pseudo-first-order manner (Figure 4A,B). For the conditions of the experiment of Figure 4A (280 μM pig muscle carbonic anhydrase III, 13 mM phenylglyoxal, and 50 mM sodium diethylmalonate, pH 7.0, 30 °C), the pseudo-first-order rate constant of inactivation for the CO_2 hydratase activity is 0.19 h^{-1}. Under similar conditions (280 μM enzyme, 28.6 mM phenylglyoxal, and 50 mM sodium diethylmalonate, pH 7.0, 30 °C), the esterase activity was lost with a pseudo-first-order rate constant of 0.45 h^{-1} (Figure 4B).

Figure 5 shows the effect of concentration on the inactivation of the CO_2 hydratase and *p*-nitrophenylacetate esterase activities of carbonic anhydrase III. Inactivation of each of the activities was dependent on the concentration of phenylglyoxal, with approximately the same concentration dependence for

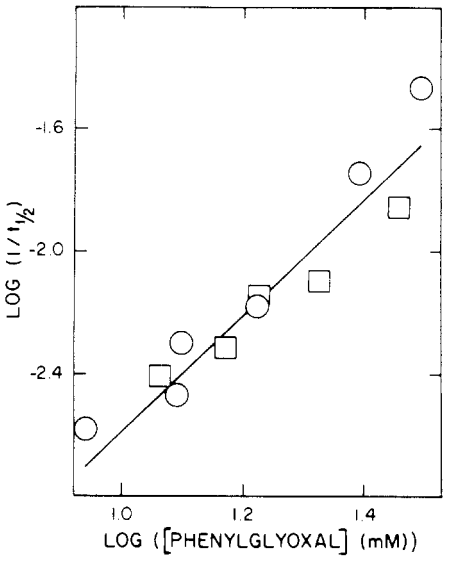

FIGURE 5: Effect of phenylglyoxal concentration on the time required for 50% inactivation of the CO_2 hydratase and *p*-nitrophenylacetate esterase activities of pig muscle carbonic anhydrase III. Reaction conditions: 280 μM enzyme and 32 mM sodium diethylmalonate, pH 7.0, 30 °C; (○) CO_2 hydratase activity; (□) *p*-nitrophenylacetate esterase activity. The data are presented as a logarithmic plot of the reciprocal of the time required for 50% inactivation against the logarithm of the phenylglyoxal concentration, as suggested by Levy et al. (1963), fitted by least-squares linear regression.

the two activities. A plot of the time required for 50% inactivation of either the hydratase or the esterase activity vs. the reciprocal of phenylglyoxal concentration (data not shown) resulted in a single straight line, with a second-order rate constant of 0.016 M^{-1} h^{-1}. A plot (Figure 5) in the manner of Levy et al. (1963) of the log of the reciprocal of the time for 50% inactivation vs. the log of the phenylglyoxal concentration gave a slope of 2.0 for both the esterase and hydratase activities of pig muscle carbonic anhydrase. This suggests that a minimum of 2 equiv of phenylglyoxal is incorporated to accomplish the loss of either of the two activities, in contrast to the response of the phosphatase activity which is lost with only one phenylglyoxal incorporated per active site equivalent.

Acetazolamide, the classic carbonic anhydrase sulfonamide inhibitor, did not offer protection of the hydratase activity against inactivation by phenylglyoxal. Unfortunately, its low solubility and low K_i made it impossible to test for its ability to offer protection against phenylglyoxal modification of the esterase activity.

Figure 6A shows the inactivation of the esterase activity with radioactively labeled phenylglyoxal. This experiment was undertaken to determine the specificity of the inactivation of the hydratase and esterase activities by the arginine modifier. The level of [^{14}C]phenylglyoxal incorporation was monitored along with the levels of the esterase activity of pig muscle carbonic anhydrase (Figure 6B). The results (Figure 6C) show that the esterase and hydratase activities are not lost with the initial incorporation of two phenylglyoxal molecules per enzyme equivalent, a stage at which the phoshatase activity is lost. Rather, the entire loss of esterase activity occurs when between about four and eight phenylglyoxals are incorporated. Precipitate appeared toward the end of the experiment when approximately 80% of the activity was lost and 7 mol of phenylglyoxal was incorporated per mol of enzyme.

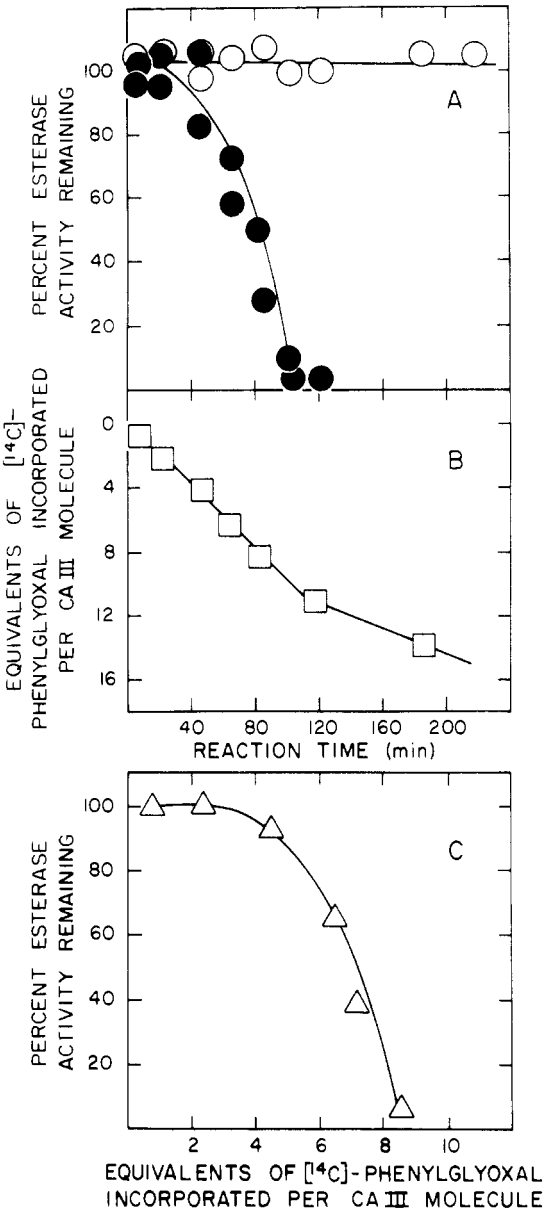

FIGURE 6: Relationship of the *p*-nitrophenylacetate esterase activity remaining and the incorporation of radioactively labeled phenylglyoxal upon modification of pig muscle carbonic anhydrase III. Reaction conditions: 280 μM enzyme, 22.6 mM [^{14}C]phenylglyoxal, and 42 mM sodium diethylmalonate, pH 7.0. (A) Plot of the percent *p*-nitrophenylacetate esterase activity remaining vs. time of reaction with phenylglyoxal: (●) in the presence of 22.6 mM phenylglyoxal; (○) control without phenylglyoxal. (B) Incorporation of molar equivalents of [^{14}C]phenylglyoxal as a function of the time of reaction. (C) Loss of *p*-nitrophenylacetate esterase activity as a function of the molar equivalents of [^{14}C]phenylglyoxal incorporated.

Discussion

Arginine modification of pig muscle carbonic anhydrase represents an example par excellence of the two categories of arginine modification which may result in the loss of enzymatic activity. In the first case, the inactivation of the phosphatase is clearly active site specific with a single phenylglyoxal incorporated for complete inactivation. In the second case, arginine modification which results in the inactivation of the hydratase and esterase activities of the pig muscle is possibly due to unspecific disorganization of the enzyme following modification of several arginine residues.

The specific inactivation of the phosphatase activity by phenylglyoxal reflects a differentiation of the active site for the phosphatase activity from that of those of the hydratase and esterase activities. If the phosphatase activity can be modified without loss of the esterase or hydratase activity—as we find in the present case—the sites cannot be identical. The

argument for nonidentical sites is furthermore supported by the differing pH–activity profiles and the difference in inhibition by acetazolamide [see Koester et al. (1981)]. The esterase activity is inhibited by the phosphatase substrate or by phenylphosphonic acid (a substrate analogue), yet both compounds are reasonable analogues of the esterase substrate as well (unpublished experiments). Indeed, phosphate (Christiansen & Magid, 1970; Bertini et al., 1978a) and the structurally similar sulfate (Koenig et al., 1980) are inhibitors of the esterase and hydratase activities of all carbonic anhydrases. It might thus be argued that the phosphatase substrate binds nonproductively to the esterase site.

Although the high reactivity of phenylglyoxal suggests enzyme affinity for the phenyl ring, a feature shared by the phosphatase and esterase substrates, an alternate explanation could be that phenylglyoxal modification occurs at a guanidinium moiety near the primary hydratase and esterase sites without blocking the binding and reaction of the esterase and hydratase substrates. If the guanidinium moiety conferred specificity for the binding and reaction of the *p*-nitrophenyl phosphate, modification of the arginine could destroy the phosphate ester binding without necessarily altering the hydratase and esterase activities. In that regard, it is interesting that hydrolysis of a similar substrate, dimethyl 2,4-dinitrophenyl phosphate, has been reported for erythrocyte carbonic anhydrase with a pH dependence similar to that of the esterase activity (Pocker & Sarkanen, 1978). Acetazolamide inhibition of the hydrolysis of dimethyl 2,4-dinitrophenylphosphatase has a K_i comparable to that of the esterase activity, suggesting that the reaction mechanism in that case involves the same well-defined substrate binding site of the esterase.

Other investigators have postulated the presence of an independent secondary site for the esterase activity which functions above pH 9 (Wells et al., 1975) and a secondary site for inhibitor binding (Verpoorte et al., 1967). The secondary site of esterase activity was found to be unaffected by as much as a 100-fold excess of acetazolamide over enzyme (Pocker & Bjorkquist, 1977). It has been claimed not to involve the metal per se (Bertini et al., 1978b) but is thought to be located close by (Lanir & Navon, 1974). Thus, the phosphatase activity might arise from binding of an esterase substrate analogue to a secondary site of esterase activity with a mechanism necessarily different from that seen for the hydratase and esterase activities at the active site zinc. If the arginine residue serves as a phosphate binding site, a histidine residue could then form the histidine–phosphate complex upon loss of the alcohol (in our case loss of the *p*-nitrophenol) as proposed by Van Etten (1982).

Whereas we obtained loss of the phosphatase, hydratase, and esterase activities upon extensive incorporation of phenylglyoxal, Tashian et al. observed *activation* of the hydratase upon modification of human CA III with butanedione (Tashian et al., 1980). These authors also reported the isolation of peptides containing modified arginine residues at positions 67 and 91 (Chegwidden et al., 1983). It must be concluded, therefore, that the two arginine modification reagents, phenylglyoxal and butanedione, react in a different manner. The phenylglyoxal molecule with its phenyl ring might have enhanced selectivity for the site which binds the phosphatase substrate *p*-nitrophenyl phosphate. Indeed, the low level of reagent required for the inactivation (4.5-fold excess over enzyme) and the 1:1 stoichiometry of incorporation argue for absolute selectivity of the phenylglyoxal for the phosphatase site. It should be noted that a 1:1 stoichiometry for phenylglyoxal modification of arginine residues in proteins is not uncommon [e.g., see Borders & Riordan (1975), Philips et al. (1979), and Myohanen & Mantsala (1980)] although the incorporation of two phenylglyoxals per arginine residue has also been reported [e.g., see Takahashi (1977a,b), Peters et al. (1981), and Takata & Fujioka (1983)] with the type of mechanism apparently being a specific feature of the respective proteins and the environment of their affected arginine residues.

After completion of the reaction of phenylglyoxal with the single arginine of the putative phosphatase active site, the enzyme is furthermore susceptible to additional modification by phenylglyoxal at multiple, unspecific sites. In contrast to phenylglyoxal, butanedione does not appear to react selectively with the site of the phosphatase activity but rather attacks (an)other site(s) and only if it is present at significantly higher concentrations (Tashian et al., 1980). It is noteworthy that a similar difference in reactivity of the two arginine modifiers was previously observed by our laboratory with regard to the inactivation of phosphoglucose isomerase (Pullan et al., 1982). These findings point to the vagaries in general of chemical modifications at enzyme active sites.

The stoichiometry and linearity of incorporation of the labeled phenylglyoxal make a strong argument against the (very remote) possiblity that the phosphatase activity of CA III might arise from an undetected, minute contamination of the carbonic anhydrase preparation by a very active phosphatase enzyme. Moreover, it is most difficult to rationalize how a 1–5% contamination could produce an exact inactivation titration of the phoshatase enzyme and do so under conditions that would fortuitously result in a precise 1:1 stoichiometry of the modifying reagent with the enzyme that would be the contaminated entity (i.e., carbonic anhydrase). In case of a 5% contamination and assuming a similar molecular weight for such a contaminating enzyme species as for carbonic anhydrase, for example, the incorporation of phenylglyoxal into the contaminant whould have to be exactly 2 equiv of phenylglyoxal at 10% activity loss, 4 equiv at 20% activity loss, etc., rising linearly to 20 equiv incorporation at 100% activity loss; this would have to occur without modification of those arginines whose integrity is required for the hydratase and esterase activities of carbonic anhydrase (see Results). The stoichiometry and kinetics of the phenylglyoxal modification of carbonic anhydrase III thus argue for a phosphatase activity that is an integral but distinct part of the carbonic anhydrase molecule.

Tashian et al. suggested that the phosphatase activity of carbonic anhydrase III might enable the enzyme to play a yet unknown role involving phosphate groups (Tashian et al., 1982). Such a function could involve phosphatase or phosphotransferase activities. Although the enzymatic rate of *p*-nitrophenyl phosphate hydrolysis is about an order of magnitude smaller than that of *p*-nitrophenyl acetate hydrolysis, the catalytic enhancement over the uncatalyzed rate by the enzyme's phosphatase activity is 2–3 times that by its esterase. The rate of transfer of the phosphoryl group to a nucleophilic compound (H_2O or some other biological substrate) may by far exceed its rate of hydrolysis (Hollander, 1971), and the stereochemical specificity of the transfer activity can be much more selective than that of the hydrolase activity (Schmidt & Laskowski, 1961). Finally, *p*-nitrophenyl phosphate may be a comparatively poor analogue of the true (as yet unknown) phosphate substrate just as *p*-nitrophenyl acetate is a poor substrate analogue for most of the serine proteases. Consequently, the slow rates observed for the phosphatase activity could simply reflect specificity effects per se. It is

reasonable to conclude, therefore, that in addition to the probable physiological role of muscle carbonic anhydrase III as CO_2 hydratase, its esterase and phosphatase activities may also be significant.

ACKNOWLEDGMENTS

We thank Dr. Michael Dunn for helpful suggestions during the preparation of the manuscript.

Registry No. Carbonic anhydrase, 9001-03-0; L-arginine, 74-79-3; phenylglyoxal, 1074-12-0.

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